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ACINETOBACTER BAUMNNII: THE USE OF POP2 AGAINST HOST DEFENSE

By

Laline Jam

A thesis

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To the Graduate Faculty:

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ACINETOBACTER BAUMNNII: THE USE OF POP2 AGAINST HOST DEFENSE

Thesis Abstract-Idaho State University (2019)

Acinetobacter baumannii is a human pathogen that is becoming increasingly well known in both the clinical and scientific fields. This gram-negative bacterium was discovered to have amorphous properties and has become a topic of concern in many clinical settings. These concerns include: the ability to form biofilms, which increases its resistance to therapeutic drugs; its high tolerance to various environments; and its ability to survive on multiple surfaces. The goal of this project was to characterize, clone, and purify a secreted serine protease present in *A. baumannii* called *Prolyl Oligopeptidase Family Protein 2 (POP 2)*. We identified this protein due to its similarity to other proteases. Further confirmation of this protease was done using automated prediction programs. Once the gene was identified, it was cloned from *A. baumannii* genomic DNA using PCR, and subcloned into the pET100 cloning vector. The ligated vector was transformed and propagated in *E. coli* TOP10 cells. Confirmed clones were transformed in the *E. coli* expression strain, BL-21. The protein expression was accomplished using an auto-induction method which produced more protein than other methods. Successful expression of the POP2 protein allowed for the purification process using Immobilized Metal Affinity Chromatography (IMAC). All processes completed throughout this project help in understanding how *A. baumannii* uses POP2 against host defenses. Results of this research can possibly lead to future advancements in targeted drug therapies against *A. baumannii* infection.

KEY WORDS: *Acinetobacter baumannii*, Prolyl Oligopeptidase Family Protein (POP2), cloning, protease, protein, purification, peptidase

Chapter I. Acinetobacter baumannii Literature Review

History

Acinetobacter baumannii was first isolated in 1911 by Beijerinck a Dutch microbiologist (1), who described it as *Micrococcus calico -acericus* (2). The genus *Acinetobacter* was later proposed in 1954 by Brisou and Prevot, as a way to distinguish the motile from the non-motile organisms of the *Achrombacter* genus (1). A comprehensive study published by Baumann et al. in 1968 lead to the recognition of organisms such as, *Moraxella lwoffi, Mima polyiroh, Neisseria winogradsjyi, Micrococcus calcoaceticu, Alcaligenes haemolysns*, and *Diplococcus mucosus,* to belong to a single genus named *Acinetobacter*. In 1971, the subcommittee on the Taxonomy of Morexlla and Allied Bacteria, officially acknowledged the genus *Acinetobacter* and in 1974, it was listed in that year's edition of Bergey's Manual of Systemic Bacteriology (1). In 1986, Bovett and Girmont used DNA hybridization to distinguish twelve DNA genospecies including *A. baumannii* (1). Over time, more and more genomic species have been discovered leading to more than 34 validly described *Acinetobacter* species to date and room for more to be discovered.

The evolution of *A. baumannii* organisms over the years has gained wide notoriety and increasing attention. The *Acinetobacter* genus are mostly ubiquitous organisms and can be isolated from most surface water and soil samples. From 2003-2005, *Acinetobacter* was the prevalent cause of infection in wounded soldiers during military operations in Iraq and was referred to as *Iraqibacter* (3). *A. nonsocmialis, A. pittii* and *A. baumannii* are the most common organisms isolated in hospitals. Currently, *Acinetobacter* is listed as an orphan disease by the FDA. Orphan diseases are conditions

which affect fewer than 200,000 people in each patient population nationwide (14). Some of these diseases have fewer than 100 people in their patient populations but, collectively, in the U.S. alone, over 20 million people are afflicted by orphan diseases(14). Because these diseases are a serious public health concern, finding treatments for orphan diseases is a necessity. *A. baumannii* has become one of the most predominant causes of nosocomial infections (infection which are acquired within a hospital setting) and a major concern in the health field (3).

Identification of the Organism

A. baumannii is a pleomorphic, non-motile, aerobic gram-negative bacillus. It is currently defined as non-fastidious, nonfermenting, catalase positive, and oxidase negative with two known genomic species. *A. baumannii* appear as short and plump and may be difficult to destain leading to easy misidentification as gram positive (1). Laboratory specimens for identification of this organism include wound swabs, sputum, bronchoalveolar lavage, cerebrospinal fluid, exudates, urine and blood (6,7). *A. baumannii* is able to grow on clinical microbiology laboratory media that is regularly used such as sheep blood agar and tryptic soy agar (TSA). Growth for this organism is best seen at 37-degree incubation temperatures. Colony morphology, when grown on sheep blood agar, is often smooth, grayish-white with the occasional appearance of mucoid colonies that are 1.5 to 3mm in diameter. *Acinetobacter* can be distinguished from other *Acinetobacter* species through metabolic testing, but can be similar to other gram-negative, nonfermenting bacterial groups like Enterococcus. The identification of the different species requires the use of molecular methods, such as amplified ribosomal DNA restriction analysis (ARDRA), high-resolution fingerprint analysis by amplified

fragment length polymorphism (AFLP), and ribotyping. One of the few methods that has been validated for *Acinetobacter* species identification and remains the reference standard, is that of DNA-DNA hybridization (4).

Infection, Disease and Spread

A. baumannii is rarely found on human skin or in human feces as part of normal flora. It is widely seen in nosocomial infections and predominantly found in moist skin areas. Hospital- acquired *A. baumannii* infections are known to colonize the oral cavity, intestinal tract, respiratory tract, and skin of infected patients. These infections are rarely seen in healthy individuals and are seldom the cause of community acquired infections but are commonly seen in immunocompromised and sick patients. In the past, *A. baumannii* has also been noted to be the most commonly identified gram-negative bacterial isolate from war wounds and the second most prevalent organism responsible for blood stream infection from extremity wounds of United State Marines during the Vietnam War (6).

The spread of this organism begins when ill patients become a reservoir of infection by shedding *A. baumannii* cells onto surrounding areas. This causes contamination of medical equipment and other surfaces. Infected cells are further carried to other areas by care takers and hospital personnel. *A. baumannii* infection can also be transmitted by patient-to-patient or through airborne transmission. *A. baumannii* has the ability to survive for prolonged periods of time on varying surfaces and has a high resistance to many therapeutic drugs and antiseptics. This organism results in frequent hospital-acquired infections caused by the bacteria that are often hard to contain. Most

hospital outbreaks and nosocomial infections caused by this organism have occurred in Intensive Care Units and have been associated with pneumonia, bloodstream infections (sepsis), and urinary tract infections (UTI's) (4).

Biofilm Formation

A. baumannii's ability to survive in various environments and its high resistance to several antibiotics can all be attributed to the organism's biofilm formation. Biofilms are defined as aggregates of self-produced exopolysaccharide matrices which surround microbial cells and the exteriors of abiotic or biotic surfaces. These biofilms allow for a higher level of protection against host immune defense, antibiotics, and harsh environmental conditions. More than 60 percent of human infections are estimated to be caused by biofilm-forming bacteria. Biofilm infections are considered chronic and require elevated doses of antibiotics to treat when compared with non-biofilm cells (7).

The leading cause of the antibiotic resistance of biofilms is poor drug diffusion which can be attributed to the accumulation of an exopolymeric matrix. Other factors that contribute to antibiotic resistance include stress, which alters the microbial genotypic and phenotypic features within the cell. When bacterial cells experiences stress, quorum-sensing signals are produced, leading to the activation of the phenotypic and genotypic structures. Activated structures aid in communication from cell to cell during changes in the environment including: temperature, quality of growth medium, acidity and oxygen level (7). When antibiotics are below the minimum inhibitory concentration (MIC), this can also be a stressor and result in the formation of biofilm in many bacterial species. Biofilm formation contributes to the tolerance of extracellular stressors of *A.*

baumannii. Many of the organisms recovered from sources like blood, urine, and sputum, showed production of biofilms. Biofilms were also reported in isolates recovered from the environment in places such as patient rooms. The environment, strain location, and ecological niche, all contribute to the capability of the bacterium to produce biofilms. While biofilm production of *A. baumannii* was most often seen in the hospital environment, it was also noted that polymicrobial bacteria produced more biofilm than those grown individually. *A. baumannii* grown with Enterococcus and Streptococcus produced more biofilm than when grown with other organisms such as *Enterococcus faecalis* (7). Biofilm formation can also be triggered through physicochemical factors such as high temperature, increase in hydrophobicity, nutrient uptake, and induced polysaccharide formation of the cell. The type and concentration of nutrients available, also play a role in biofilm formation. Higher concentration of nutrients decreased biofilm formation due to decreased competition among other bacterial isolates and detachment. Poor nutrition in contrast, increased biofilm production. High iron concentrations correlated with an increase in antibiotic resistance. Agitation of organism affected the elasticity of biofilms. Electrostatic interaction allowed for bacteria to attach tightly and rapidly to positively charged surfaces and the pH and ion content determined the strength of bacterial attachment to surfaces. Just as biofilm formation plays an integral role in the strength and resistance of *A. baumannii*, its secretion system also plays an integral role in accomplishing host infections.

Secretion

A. baumannii infects its host using a secretion system. Secretion systems are utilized by many bacterial pathogens and use a variety of methods to invade hosts, suppress the immune response, and result in damaged tissue (10). There are two main types of secretion systems that have been studied in *A. baumannii*. A type II (T2SS) and a type VI (T6SS) (10). The Type II secretion system is a two-step process and is widespread among gram negative organisms that are capable of living in various conditions.

The first step of this system occurs when the terminal secretion signal of a protein is translocated by a general secretory pathway across the inner membrane. In the extracellular space, proteins are secreted by the T2SS after the removal of the secretion signal. T2SS channels are only found on the outer membrane, therefore, proteins must be presented to the periplasm through the Sec or Tat secretion pathways. This allows substrates to be transported across the inner membrane.

The T6SS, also known as the type VI secretion system, is a secretion complex with multiple components that lead to the deadly injection of protein toxins into other bacteria. Many gram-negative pathogens are aided by T6SS in creating a favorable niche through the elimination of competing bacterial organisms. This machinery consists of thirteen core structural proteins that are encoded in a single genetic locus and are often conserved across bacterial species. The T6SS attacks the target by injecting toxins into the cell. This leads to the interference of important cellular processes which ultimately lead to bacterial killing. The two major proteins secreted by T6SS are hemolysin co-

regulated protein (Hcp) and valine glycine repeat protein (VgrG). These two proteins are used to pierce, deliver, and inject effector proteins and together form the capping spike and tail tube. *A. baumannii* is known to use both the T2SS and T6SS secretion pathways when infecting the host and evading its defenses (10).

Antibiotic Resistance

A. baumannii has a multitude of beta-lactam resistant strains and mechanisms for antimicrobial resistances. These strains illustrate the ability of the organism to respond quickly to alteration of specific environmental pressure. Inclusion of external factors and upregulation of natural resistance processes are all attributes that have made *A. baumannii* an organism of much interest. One of the mechanisms of antibiotic resistance in this organism is enzymatic degradation by beta-lactamases. As noted, due to the complexity of *A. baumannii*, there are various mechanisms that may often work together to produce the same outcome. The chromosomes of all *A. baumannii* strains inherently encode *Acinetobacter*-derived cephalosporinases (AmpC). Overexpression of the AmpC is regulated by an upstream element called ISAba, which is seen frequently in correlation with increased AmpC gene resistance to extended spectrum cephalosporins. The stability of cefepime and carbapenems appear to be unaffected in response to both enzymes. Ambler class A group of extended-spectrum beta lactamases (ESBLs) have been described for *A. baumannii* (1). The beta-lactamases with carbapenemase activity which include the serine oxacillinase, are the most concerning (1).

An important genetic marker in identification of *A. baumannii* organism to species level, comes from the intrinsic class called D oxacillinase. D oxacillinase belong

to the OXA-51-like group of enzymes that have a weakened ability to hydrolyze penicillins and carbapenems. Production of oxacillinases encoded by genes of linkages such as blaOXA-23, blaOXA_40 and blaOXA-58 are the most common enzymatic mode of carbapenem resistance.

Nonenzymatic mechanisms to Beta Lactam resistance

Nonenzymatic mechanisms, such as the alteration in outer membrane proteins, has been attributed to carbapenem Beta lactam resistance along with changes in expression or the affinity of protein binding penicillin. *A. baumannii* meropenem and imipenem resistance is associated with the loss of a membrane porin called CarO. Other forms of non-enzymatic pathways of *A. baumannii* associated with multidrug resistance includes a resistance-nodulation-division (RND) family-type pump called AdeABC, which due to point mutations, lead to over expression of the pump. Multidrug resistance and loss of pump function have been associated with the insertional inactivation of transmembrane components of the AdeABC pump.

Aminoglycosides and Quinols

Multidrug resistant *A. baumannii* strains have a high prevalence of aminoglycoside-modifying gene coded enzymes in class I integron. The rRNA methylated strains of *A. baumannii* have been found in Korea, Japan and the United States. The resistance mechanism for this strain is becoming more common and includes the impairment of the aminoglycosides target binding site. This results in a high level of resistance to all aminoglycosides that are clinically useful. These aminoglycosides include amikacin, tobramycin, and gentamicin. Quinols are another drug class effected

by modification mechanisms. In quinols, the DNA gyrase or topoisomerase IV modification via mutation interfere with target binding sites in *A. baumannii* that in turn affect quinolones, which are substrates for multidrug efflux pumps.

Tetracycline

Tetracyclines and derivatives can show resistance when mediated by efflux or ribosomal protection. Efflux pumps specific to tetracycline encoded by tet(A) and tet(B) determinants have been found in *A. baumannii*. The tet (A) is associated with resistance to tetracycline but not minocycline. Multidrug efflux systems including the AdeABC pump, make this class of antimicrobials less susceptible to efflux. Further confirmation for this mechanism id done through molecular methods.

Other Methods

Unknown mechanisms of resistance for polymyxins in *A. baumannii* are still being investigated. Other resistant mechanisms for *A. baumannii* show the association with integrons and their multidrug resistance phenotype is present among many strains. Conserved regions of the intergons contain a gene that contributes to antiseptic and sulfonamides resistance which may also be heightened by efflux (1).

In some European countries, the spread of multidrug resistant *Acinetobacter* infection has occurred on a national scale. The pathogen is no longer confined to inner city hospitals, but instead, has spread through interhospital patient transfers. New York hospitals also experienced outbreaks of carbapenem-resistant *A. baumannii in* 1991 and 1992. During 2002-2004, approximately 50 European hospitals reported that 73.1% of *A.*

baumannii isolates were susceptible to meropenem, 69.8% were susceptible to imipeme,32.4% to ceftazidime, 32.4% to ciprofloxacin and 47.6% were susceptible to gentamicin. Approximately 75 centers across the U.S. collected isolates of *A. baumannii* where 60.2% had susceptibility to imipenem. Other countries in Asia and the Middle East have documented various outbreaks of multidrug resistant *A. baumannii* (2).

Treatment

Mechanisms that contribute to *A. baumannii* antibiotic resistance, include enzyme degradation, outer membrane modifications, efflux mechanisms, and alteration to binding sights. This leads to a high degree of difficulty when choosing the right therapeutic treatment for infections caused by this organism. With the high probability that patients with *A. baumannii* related illnesses would show resistance to first-line drugs, all infection treatments should be performed after a complete analysis of susceptibility testing. Although a thorough look at susceptibility testing before treatment is ideal, this may lead to a delay in patient treatment, and result in a higher likelihood of worsening patient conditions.

Regardless of the probability of increased infection, the most common drug of preference for serious *Acinetobacter* infection is imipenem, from the class of carbapenems. The use of this drug as an immediate response to infection, may be useful initially, but may make it less effective in future treatments (2). Therapies that could be utilized in the years to come include bacteriophage, bactericidal gene transfer therapy, cathelicidins, radioimmunotherapy, phototherapy, and nanotechnology.

We are choosing to do this project in the hope that the results from characterization, cloning and purification of the *pop2* gene will help us gain a better understanding about *A. baumannii* and help in the advancements of drug therapies and treatments.

Prolyl Oligopeptidase

Prolyl Oligopeptidase, a family of serine proteases, are enzymes which are able to hydrolyze peptide bonds located next to proline residues. This results in protein breakdown. These enzymes are widely distributed in various types of gram-negative bacteria including *A. baumannii*. Just like biofilms, this secreted serine protease contributes to the evasion of host defenses and spread of *A. baumannii* (11). One mechanism of action for these secreted proteases includes downregulating the cascade response through the degradation of complement components and regulators. Additionally, the secreted protease contributes to the resistance of this pathogen, by breaking down host antibodies, which hinder the host's ability to fight the infection. Overall, serine production, as a result of antibody digestion, makes it easier for bacteria to spread toxins to other species (11, 12).

Chapter II. Characterization, Cloning, and Purification of POP2

MATERIALS AND METHODS

A. baumannii infection is an increasing concern in the clinical field. From this organism's classification, its molecular components, its ability to be resistant to varying environments, and a wide array of therapeutic drugs, makes this organism both unique and concerning. This organism was found because of our interest in orphan diseases. Knowing that *A. baumannii* infection is considered an orphan disease, we speculated that it harbored some type of proteases for self-defense which it used as a mechanism to evade host defense. Because *A. baumannii's* genome was readily available, we used the finding from previous lab work of a known secreted protease (DPP4) to find a candidate protease. In addition to using molecular methods and laboratory techniques, the goal of this project was to identify, characterize, clone and purify the serine protease present in *A. baumannii* called the Prolyl Oligopeptidase Family Protein (POP2).

Identification of POP2 and Primers

To begin this process, bioinformatics tools (like Expasy.org and Pfam database) were used to determine the molecular weight and pI of POP2. A catalytic triad for the active site was then predicted and comprised of serine, aspartic acid and histidine. Based on the retrieved gene sequence, three primers were designed, which covered the 5' and 3' ends of the gene (figure 1).

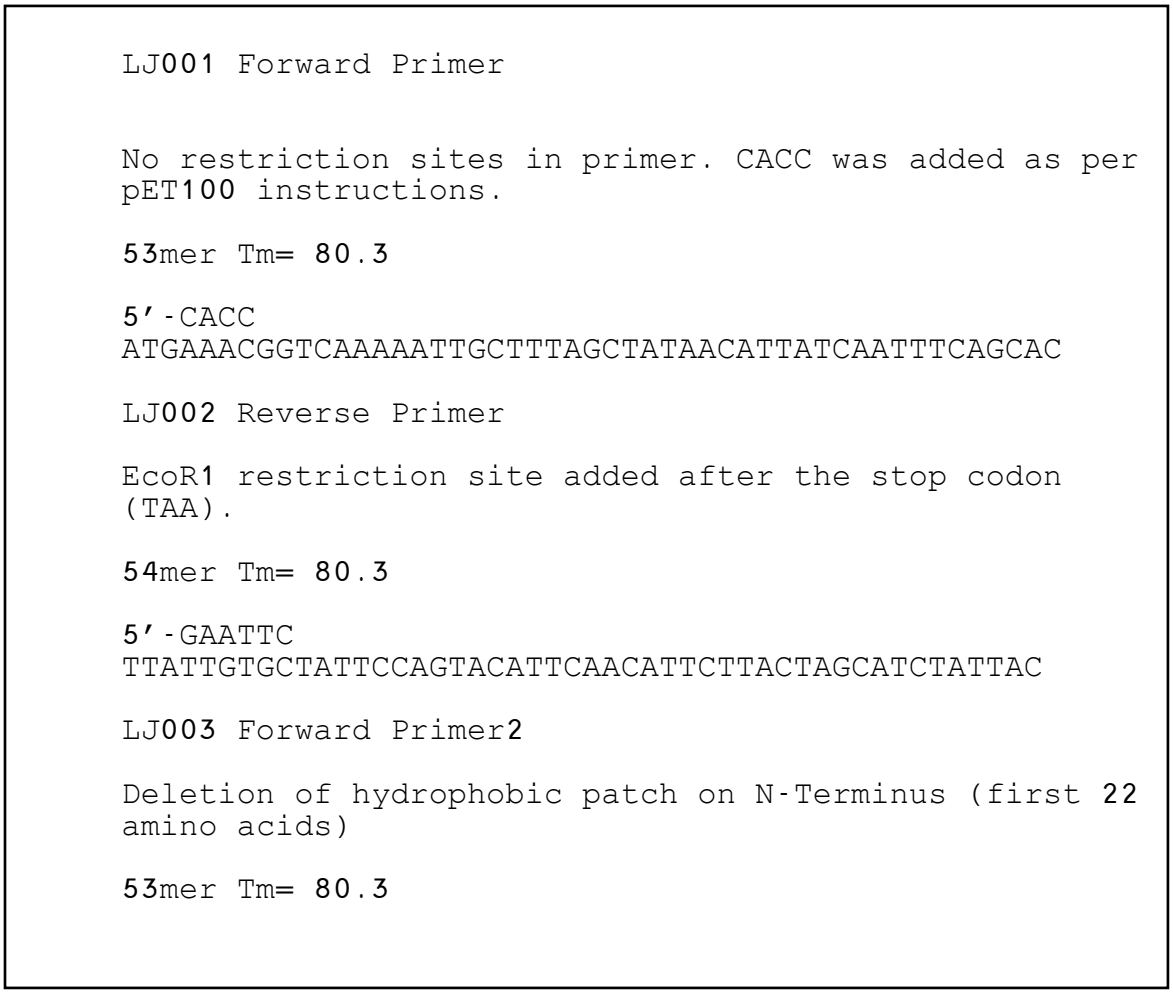

Figure 1 The primer sequences created for cloning *pop2*. Forward primers LJ001 and LJ003 with one reverse primer LJ002. LJ001 (made with the first 16 amino acids of *pop2* gene) included a hydrophobic region while LJ003 deletes the hydrophobic region by not including the first 22 amino acids. LJ003 primer includes the terminal amino acids 421-435 of the protein. The melting temperature of all primers was 80.3 °C for consistent PCR conditions.

The primers were located on the 5’ and 3’ ends of the gene, in order to optimize cloning: LJ001, a forward primer annealed to the 5’ end of the gene and is located within the hydrophobic region, LJ002; the reverse primer, which annealed to the 3’ end of the gene; and LJ003, a forward primer that annealed to a position away from the hydrophobic region. Because this protein has a known hydrophobic N-terminus, which corresponds to the secretion tag, we designed the forward primer LJ003, to start at amino acid 23 in order to clone around the region. Primers were used along with two polymerases and a DNA template under optimized polymerase chain reaction (PCR) conditions (table 1) to amplify the DNA.

PCR Conditions and Polymerases

Table 1. Optimal PCR conditions as used for the amplification of POP2 DNA.

Phase	**Temperature**	**Time**
Cycle 1	95^{o} C	3 min
Cycle 2 (x 30)	95 ^{o} C	30sec
	55 ^{o} C	30sec
	72 ^{o} C	3min
Cycle 3	72 ^{o} C	3 min
	4 ^{o} C	Infinite

The PCR process required the continuous separation of *pop2* DNA strands, annealing the DNA strands to primers, and extending of *pop2* DNA using a polymerase. GoTaq and Phusion polymerases were both tested, and it was determined that Phusion polymerase was the most effective. The presence of a DNA band, at approximately 1.3 kbp on the agarose gel using Phusion polymerase, demonstrated it was the preferred polymerase over the GoTaq polymerase (figures 2 and 3.).

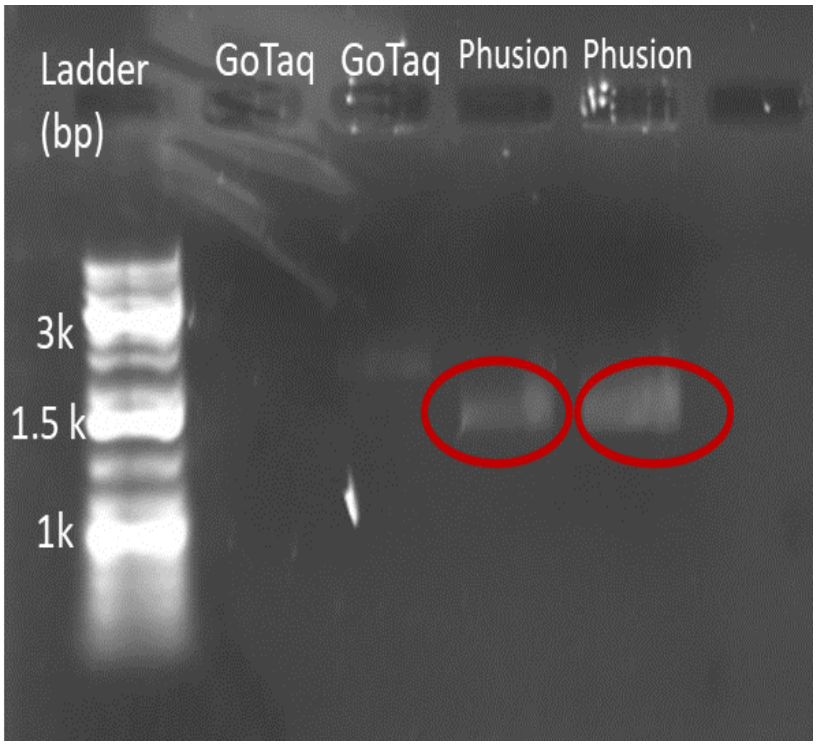

Figure 2. A 1% agarose gel, stained with ethidium bromide, demonstrated the presence of the correctly sized bands (1.5 kbp, circled in red) produced by Phusion polymerase. GoTaq produced larger bands. Phusion DNA polymerase was determined to be the better polymerase to use for this project.

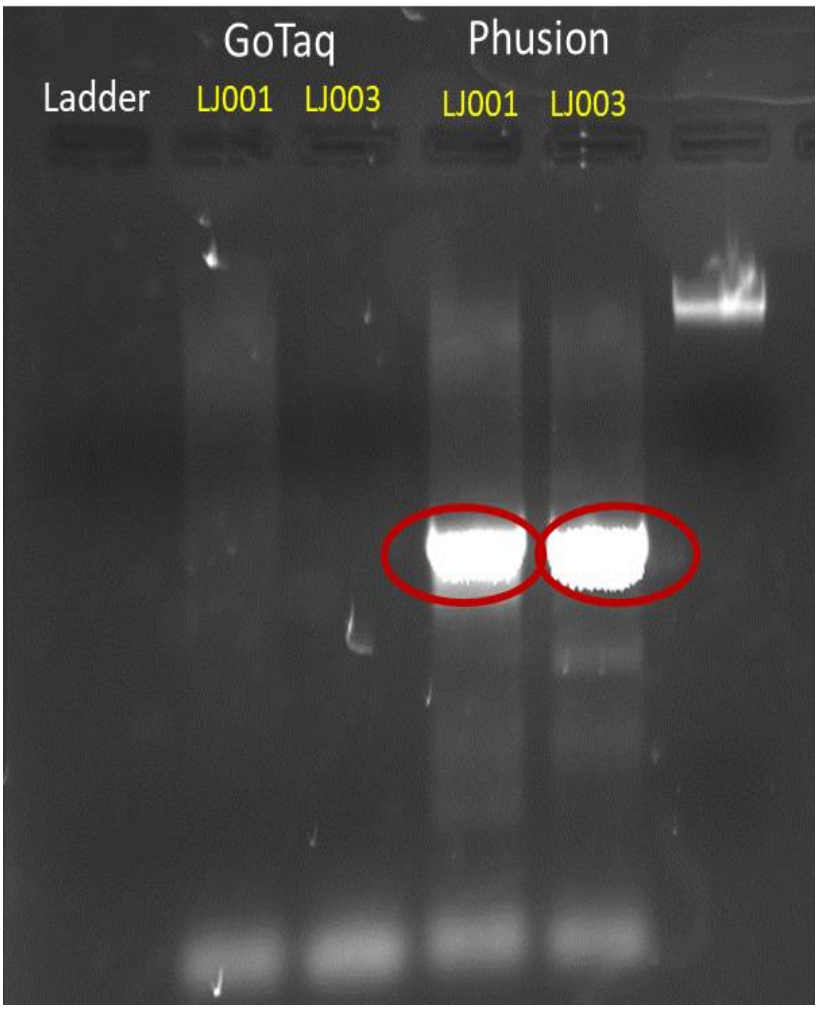

Figure 3.2. A 1% agarose gel, stained with ethidium bromide, demonstrated bands produced by the Phusion polymerase. Each of the two lanes was ran with varying forward primers, LJ001 with the hydrophobic end and LJ002 without the hydrophobic end. The band shown under Phusion and primer LJ003 lane, is slightly bigger and brighter. This indicates a higher yield of DNA, therefor Phusion polymerase with forward primer LJ003 would be more effective for this project.

Generation of the expression plasmid, pET100_POP2

DNA ligation was accomplished by using the PCR amplified gene and a pET100 vector (figure 4.2). The pET100 vector is a multifunctional plasmid which allows cloning and expression within one plasmid. We inserted the POP2 gene into the pET100 plasmid which was transformed into *E. coli* TOP10 cells for further plasmid production. The POP2 gene-pET100 construct, along with the *E. coli* TOP10 cells, was gently mixed before placing on ice for 20 minutes. Cells were heat shocked at 42 °C for 45 seconds then placed on ice for 2 min. 500 uL of LB (Luria broth) was added to the transformation reaction and inverted to mix. The solution was placed at 37 °C for an hour to allow for cell recovery. The *E. coli* mixture was plated on LB ampicillin agar plates and inoculated overnight at 37 °C.

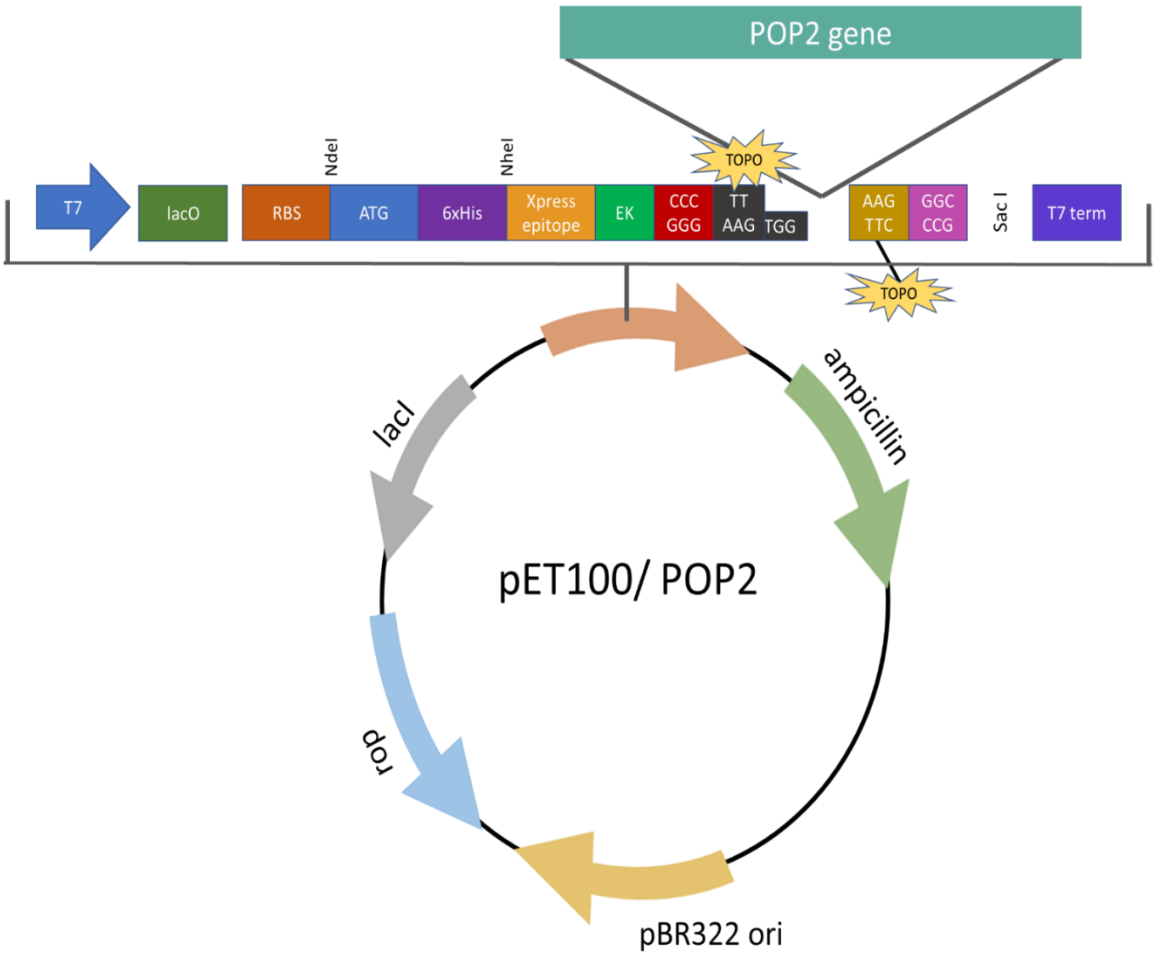

Figure 4.- The pET100-POP2 cloning construct. This construct contains a T7 promoter with a lac operon operator and a T7 terminator. The *pop2* gene is inserted into the multiple cloning site of the pET100-POP2 cloning construct.

Cloning the *pop2* Gene

Colonies from the transformation were screened using PCR. Primers LJ001 and LJ003 were used in a master mix for PCR colony screening. The master mix contained 0.2 uM of each individual forward primer with 0.2 uM of reverse primer, along with a 1X concentration of 5X green GoTaq buffer, 0.2 uM of 10mM dNTP, GoTaq polymerase and dH20 for a total volume of 725 uL of each mix. 24.5 uL aliquots of the master mix where placed into labeled PCR tubes and inoculated with each respective screening colony. After completion of PCR, each product was run on agarose gel to determine which clone was positive for the gene (figure 5). A mini prep kit was used to prepare DNA for sequencing to further confirm the clones. Glycerol stocks of clones were prepared and stored in the -80 °C freezer for future use. All clones were sent to the Molecular Research Core Facility (MRCF) for sequencing. The three clones named 3A, B1, and B3 were had the least errors and best match with the original *pop2* gene. The B3 clone was selected to proceed with protein expression.

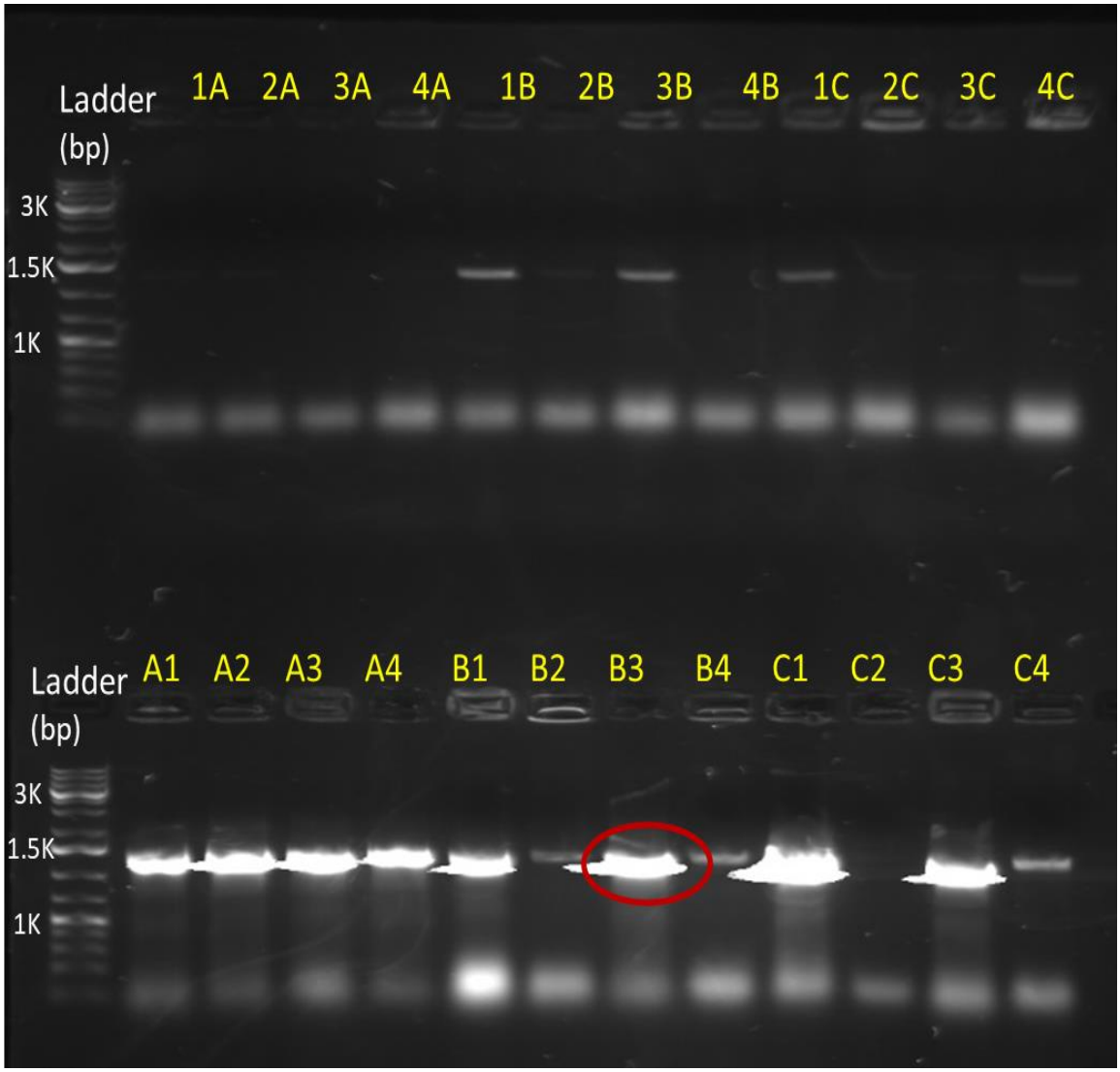

Figure 5. pictured above is a 1% agarose gel stained with ethidium bromide. This shows the PCR product of all of the screening colonies. The top half of screening colonies labeled: 1A, 2A, 3A, 4A, 1B, 2B, 3B, 4B, 1C, 2C, 3C, and 4C all were used with GoTaq polymerase. Although some faint bands can be seen just below the 1.5 K bp mark for clones 1B, 3B, and 1C, none of the clones were used. The bottom half of the gel used screening clones labeled A1, A2, A3, A4, B1, B2, B3, B4, C1, C2, C3, C4 all used Phusion polymerase and yielded the best results. All samples (excluding C2) from the Phusion clones were sent to the MCRF for sequencing. Clone B3 (circled above in red) was proven to have the highest percent identity to the original POP2 DNA at 89.9%. Both primers were used to optimize the selection of the best clone for sequencing.

Protein Expression and Purification

Protein Induction

After identification, the B3 clone was transformed into the protein expression E. coli strain, BL21. By taking advantage of the cloning construct (refer to figure 4), the first step in the purification was to induce E. coli to make protein by appending a poly-histidine tag to the N-terminus of the protein. Immobilized metal affinity resin, which uses modified nickel or cobalt silica support to capture can be used to capture proteins with a poly-histidine tag. To induce protein expression, the auto-induction system Studier was used [13].

The Studier Auto-induction method, used to prepare the necessary amount of protein for activity studies, contained the following: 505S, solution M, MgSO4, mixed metals, antibiotics, and LB media. All solutions were mixed together to create an auto-induction media which was inoculated with an overnight culture of POP2. Cells were left to incubate for 12 to 18 hours at 37 °C and harvested by centrifugation at 5,000 xg. The pellet was resuspended in resuspension buffer containing HEPES, lysozyme, DNase 1, and 2-mercaptoethanol (BMe) and was frozen at -20 °C until the next step.

Protein Solubilization

The cells were freeze-thawed three times, then the cell extract was separated by centrifugation at 20000 xg. The amount of protein was checked by a 12% SDS-PAGE gel. It was observed that the protein was mostly insoluble and was most likely confined to inclusion bodies. The presence of protein in the cell debris pellet indicated that the protein existed as a precipitate and this procedure would need to be improved. After

many iterations, the procedure was optimized to resuspend the cell pellet with buffer plus leupeptin, which is a reversible protease inhibitor. After the cells were resuspended in a resuspension buffer that contained HEPES buffer, lysozyme, and 100µL of leupeptin, the solution was placed in the -20 °C freezer overnight. The solution was thawed and centrifuged the next day. A 30µL sample of both the sup and pellet were taken to be examined via a 12% SDS gel. Conformation of protein in the solution lead to the purification through the use of a nickel-based His-Link resin.

Purification

10 mL of the solubilized protein was added to 2 mL of washed resin slurry and gently rocked for one hour at room temperature. The resin was allowed to settle, and the supernatant was decanted. The resin was washed three times with washing buffer containing 50 mM HEPES (pH 7), 50 mM KCl, and 0.5 mM 2-mercaptoethanol (BMe). To elute the bound protein from the column, 5 mL of elution buffer containing the wash buffer plus 0.5 M Imidazole was added to the resin and allowed to incubate for 20 minutes. The protein solution was decanted and further purified using dialysis. The dialysis buffer contained 50 mM HEPES (pH 7) 50 mM KCl, 1 mM BMe, and 20% glycerol, and was placed overnight at 4°C. The presence of the protein in the elution fractions after dialysis was confirmed by a 12% SDS-PAGE gel, stained with Coomassie Blue.

Chapter III.

RESULTS

Transformants were PCR screened using forward and reverse primers LJ003 and LJ002. Clones A1, B1, B3, were determined to have 57.9 %, 79.8%, and 89.9% identity respectively, to the known POP2 gene, which is comprised of 1314 nucleotides (refer to figure 6a and 6b for the full DNA and protein sequence alignment). B3 was the clone with the highest similarity and lowest number of errors and was chosen for transformation into BL21 E coli cells and protein production. The induction of this protein this was effective because the pop2 gene is under control of the T7 promoter and lactose operator. The presence of a protein band, around the 46 kD ladder, on a 12% SDS gel demonstrated that the induction was successful and protein expression was effective. This experiment indicated the best expression was after 2 hours of induction. Although induction using IPTG proved to be a feasible method of POP2 protein expression, a later method of auto-induction demonstrated to be even more effective and was used for all protein production.

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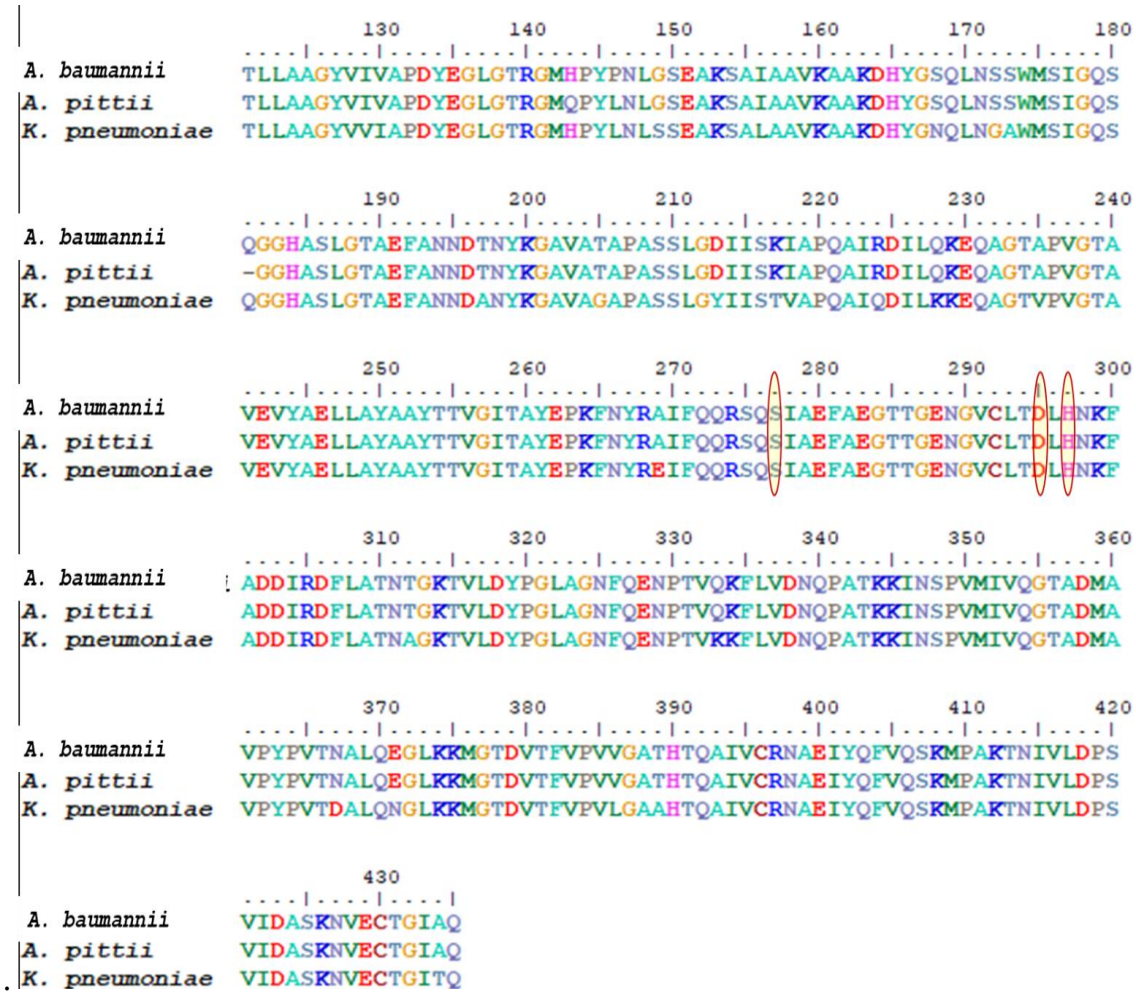

Figure 6a. The protein sequence and predicted catalytic triad, serine, aspartic acid, and histidine, all denoted by the amino acid abbreviation S, D, and H respectively. Predicted location was S-277, D-295, and H-297. This was conserved throughout the alignment of *A. baumannii*, *A.pitti*, *and K. pneumoniae*. The alignment generated using BioEdit software and the CLUSTALW algorithm.

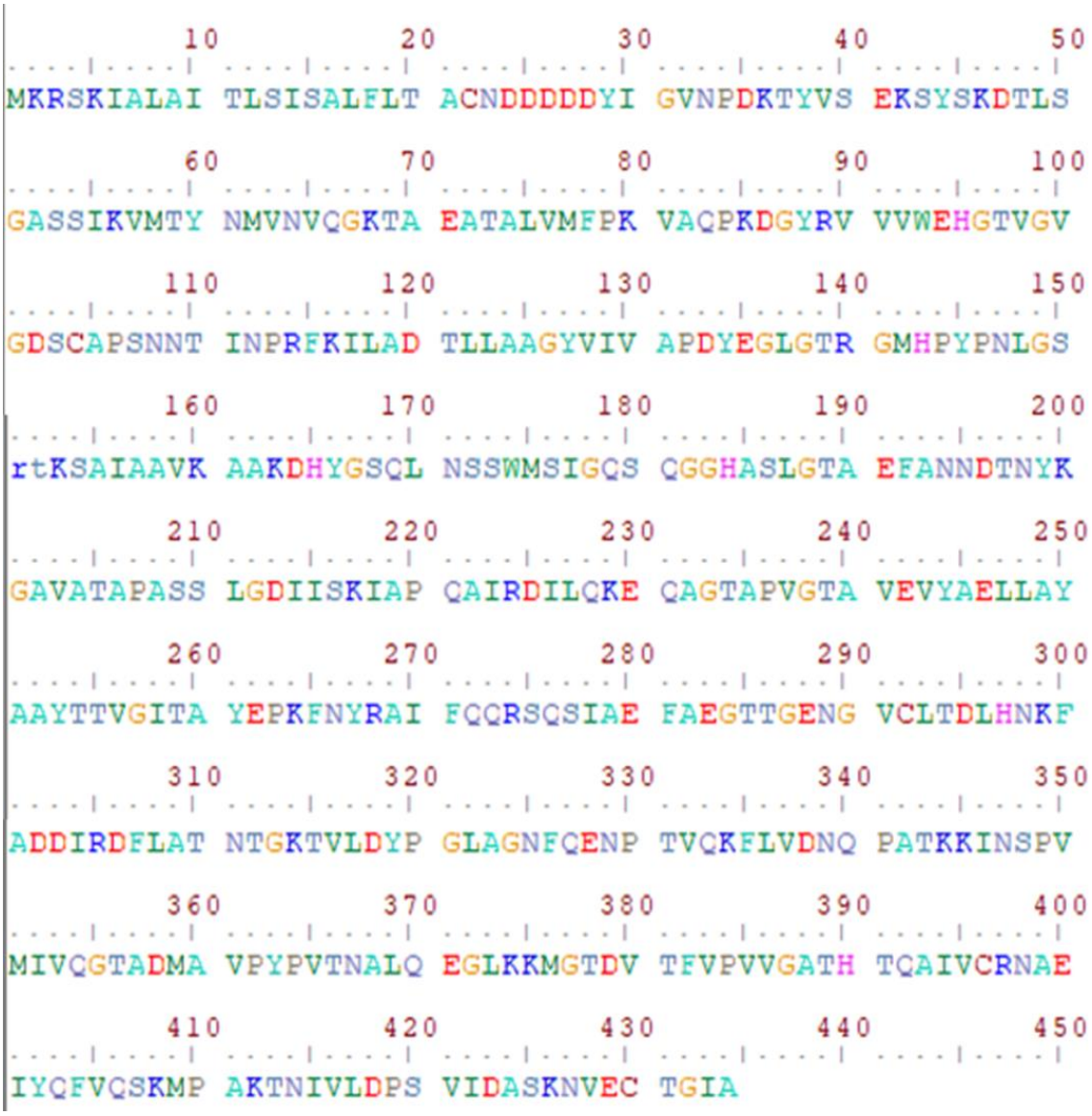

Figure 6b. The protein sequence of POP2 from *A. baumanniii*

POP2 Protein Properties

POP2 is composed of 438 amino acids and has a molecular weight of 46,4349.45 Daltons or 46.4 kD. The isoelectric point (pI) of POP2 is calculated to be 5.1. Major protein classes identified in this protein were the AXE1 superfamily, located at 70 to 210 amino acids, the Abhydorlase superfamily (lipase) located at 122 to 391 amino acids, and the Abhydrolace_4 located at 342 to 411 amino acids. The active site of this gene was predicted to be located within the Abhydrolase superfamily (Lipase) with the catalytic triad amino acids, serine, aspartic acid, and histidine, located at 277, 295, 297, respectively (figure 7).

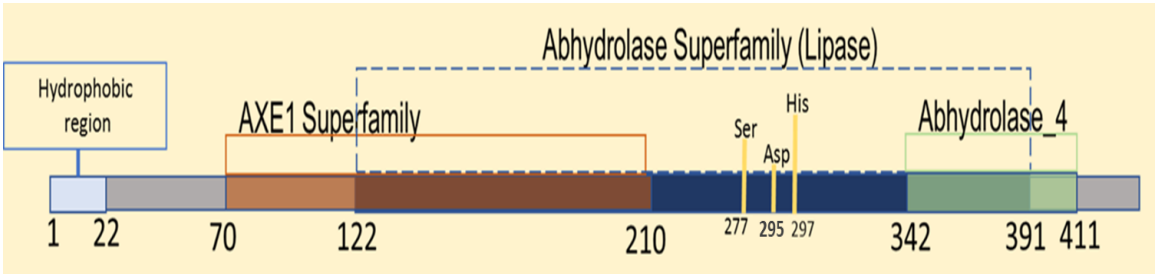

Figure 7.3. A protein map of POP2 with the catalytic triad amino acids, aspartic acid, histidine, and serine shown in yellow. The acetyl xylan esterase1 (AXE1) superfamily catalyzes hydrolysis of acetyl groups. The Abhydrolase-4 family contains an alpha/ beta hydrolase fold. A potential trypsin site was predicted to be close to the C- terminus, around 300-360 amino acids.

After protein expression was induced, the cells were lysed using three freeze thaw cycles and the resulting solution was centrifuged to separate the cell debris and soluble protein. POP2 protein localized to the pellet. Solubilization of this protein was proven to be an exceptional challenge. Urea, which is classically used to solubilize hard to solubilize proteins, was used first. Samples of the POP2 pellet were resuspended with 1

M, 2 M, 4 M and 8 M urea and incubated at room temperature for one hour. The 4 and 8 M urea was the best at solubilizing the pellet as seen in figure 8.

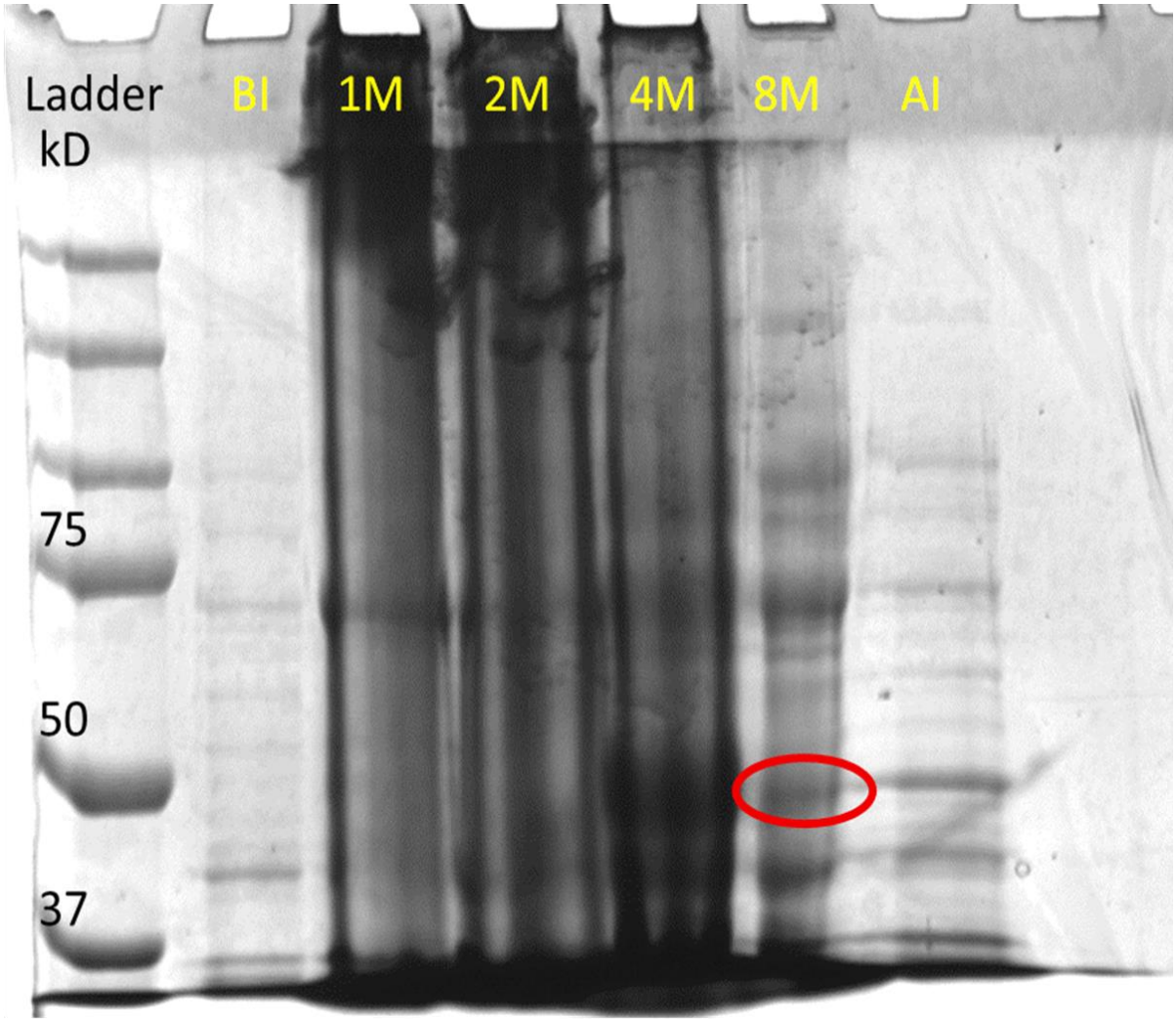

Figure 8. A 10% SDS-PAGE gel, stained with Coomassie Blue, shows the results of urea solubilization. Concentrations of 1 M, 2 M, 4 M, and 8 M urea were used. The gel demonstrated that the protein is in the 4 and 8 M urea solution, as noted by the red circle corresponding to the band right below 50 kDa.

While urea worked well, it was not the best method because proteins treated with urea need to be refolded. Based on results in the lab using the protease DPP4, proteins with hydrophobic character could also be solubilized using an excess of buffer. A 0.200-gram POP2 pellet was added to 20 mL of HEPES pH 7 resuspension buffer and was

incubated at room temperature for 2 hours (longer incubation times were also used). This method was also successful as bands were seen on the 12% SDS-PAGE gel (see figure 9).

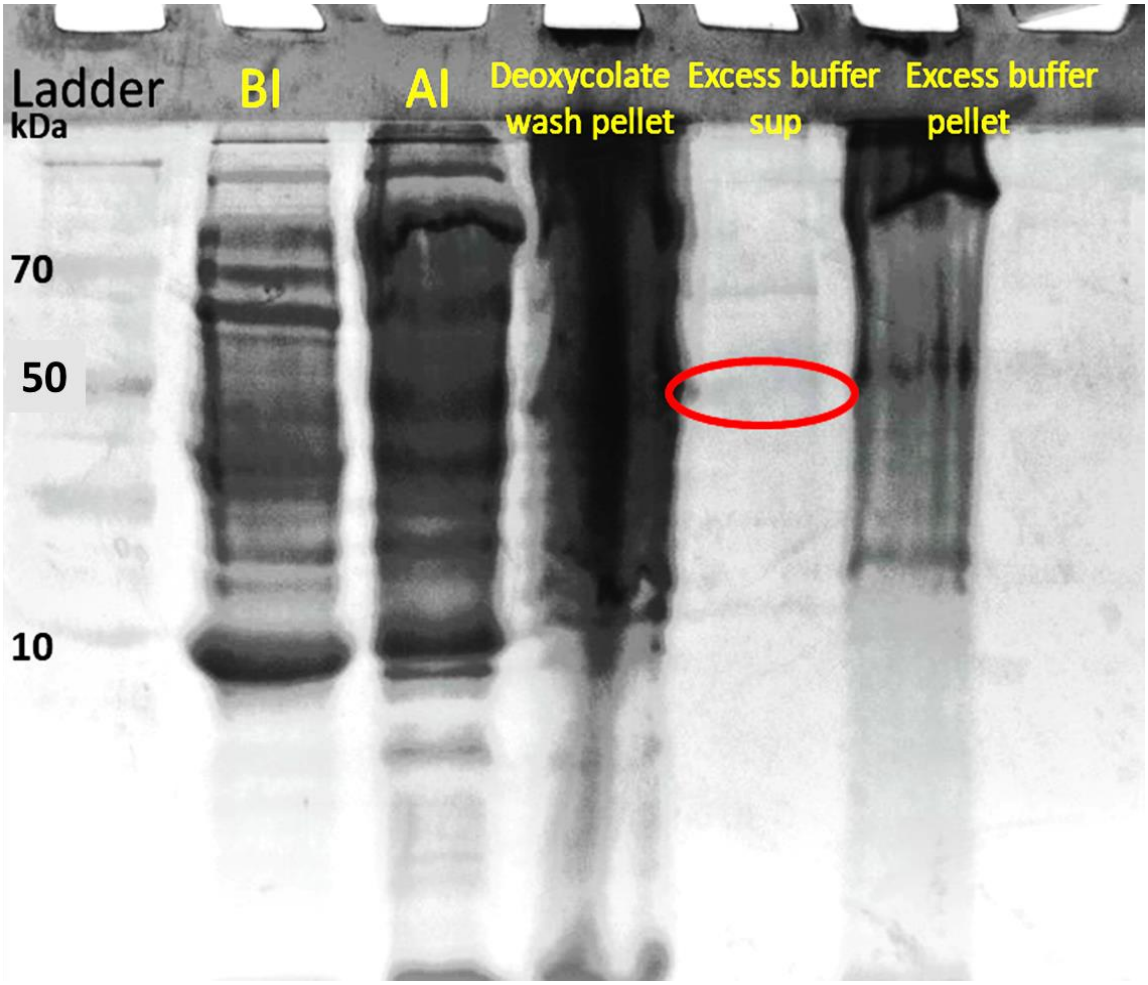

Figure 9. A 12% SDS-PAGE gel, stained with Coomassie blue, showing the results of an attempt to solubilize the protein pellet through the use of excess buffer. This method was successful as the presence of a very faint band can be seen circled in red, at the corresponding protein ladder bellow 15kDa. Although successful, the concentration of protein using this method is too dilute and not ideal for further protein analysis.

Further reading in the literature also reported that purification of proteases was better accomplished by putting in reversible protease inhibitors into the cell extract. This was tried using leupeptin, a reversible serine protease inhibitor. Using leupeptin, more

POP2 was found in the supernatant (after cell lysis) and this was the most practicable method for getting the protein in the pellet to go into solution (see figure 10).

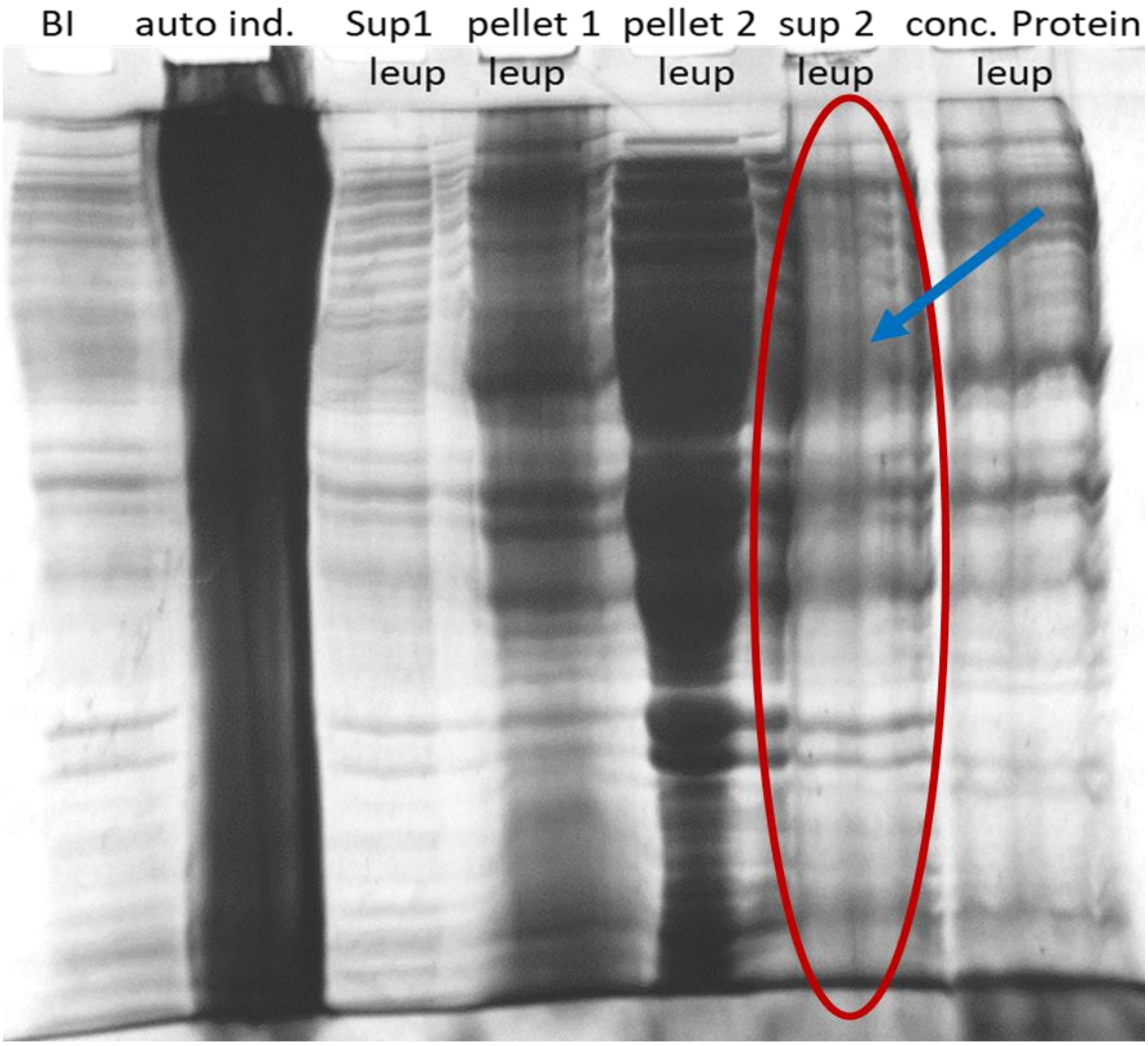

Figure 10. A 12% SDS-PAGE gel showing the presence of protein in both the leupeptin supernatants and pellets. Solubilization of the protein pellet was successful by using leupeptin, a protease inhibitor, which placed more protein into solution. This proved that solubilization using leupeptin was a more ideal method for this project. Blue arrows show suspected POP2 protein bands.

Nickel-based IMAC resin was tested and used to purify the protein solution. It was determined that the nickel-based resin was better because the purified protein yield was higher than other purification schemes. Repetition of the process also lead to the use of dialysis after IMAC purification, to further purify the protein solution and exchange the buffer. The presence of a single band on a 12% SDS-PAGE gel, from the purified sample, proved that the POP2 protein was successfully purified (refer to figure 11).

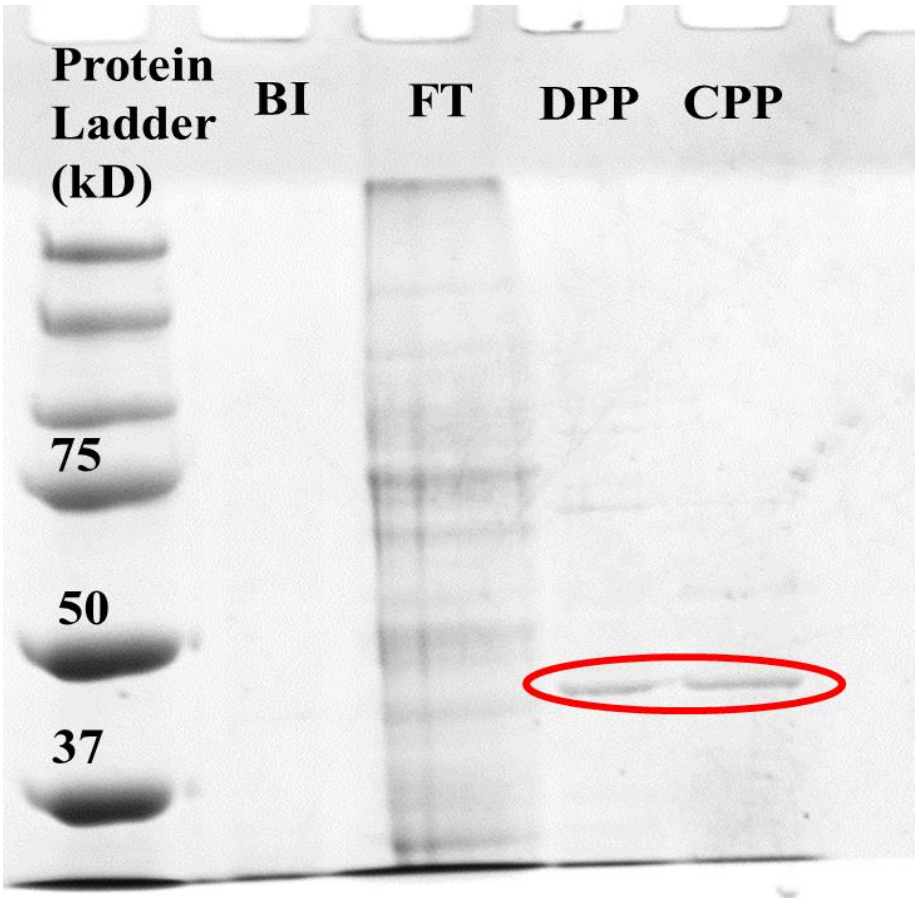

Figure 11. A 10% SDS-PAGE purification gel, stained with Coomassie Blue, demonstrated both the diluted purified protein (DPP) and the concentrated purified protein (CPP) bands are pure. The POP2 protein, the 46 kD protein bands, are circled in red.

DISCUSSION AND CONCLUSION

A. baumannii is an adaptable bacterium that has robust characteristics that contribute to its strength. This bacterium forms biofilms and has secretory mechanisms that make it a point of concern in many fields but is most alarming in the medical field. The ability of this pathogen to form biofilms contributes to its high and growing resistance to various surfaces and to antibiotics. In an attempt to further understand *A. baumannii*'s ability to evade hosts' defense, we cloned and purified one of its secreted serine proteases, POP2. By identifying, purifying, characterizing, and testing POP2, we gain insight on how protein activity aids this organism and adds pertinent information to the body of knowledge.

Working with this protein posed many challenges. Obtaining the proper sequence took multiple attempts and alignments. Because this protein was a protease, proper timing in purification was an integral factor the success of this process. The induction process was repeated multiple times and although often effective, the protein was often degraded after the freeze-thaw cycle, most probably due to protein activity. The auto-induction was the protein production method of choice and produced better protein expression than the induction method using IPTG, even though it took a longer period of time.

Once the protein was expressed, its hydrophobic nature proved difficult to manage during purification. Although solubilization with 8 M urea was effective, the protein could not be properly refolded and was not active after several attempts to perform activity assays using a casein. Solubilizing protein in excess buffer worked, but

it left the protein too dilute and hard to concentrate which was necessary to perform activity assays.

The initial intention for the use of leupeptin was to prevent POP2 from degrading during purification. However, leupeptin helped increase the protein solubility in solution and allowed for the purification. These results are shown in figures 10 and 11. Leupeptin was most likely able to solubilize the protein pellet because it inhibited POP2's self-protease activity, which is a common mechanism of activation for human-pathogen proteases.

Future Direction

Overall, the identification, cloning and purification of *A. baumannii's* Prolyl Oligopeptidase Family Protein (POP2), was successful. Using the results of this research, further identification and evaluation of the activity of this protease could be accomplished, leading to new and improved advances for drug therapies and treatment of the diseases caused by this organism.

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