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Is approved by the final examining committee:

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Approved by: Simon Atkinson 06/26/2013
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# INVESTIGATION OF THE POTENTIAL BACTERIAL PROTEASOME HOMOLOGUE ANBU

A Thesis

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| | |
|---|---|
| °C | Degrees Celsius |
| 19S RP | 19S Regulatory Particle |
| 20S CP | 20S Core Particle |
| ATP | Adenosine Triphosphate |
| BPH | β-proteobacteria Proteasome Homologue |
| DMSO | Dimethyl Sulfoxide |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic Acid |
| HslV | Heat-shock Locus Protein |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| Kan | Kanamycin |
| kDa | Kilodalton |
| LB | Lysogeny Broth |
| μL | Microliter |
| MDa | Megadalton |
| mL | Milliliter |
| mM | Millimolar |
| Mpa | Mycobacterial Proteasome ATPase |

| | |
|---|---|
| NTA | Nitrilotriacetic Acid |
| nm | Nanometers |
| PA14 | *Pseudomonas aeruginosa* Strain 14 (Wild Type) |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PAN | Proteasome-Activating Nucleotidase |
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylene glycol |
| pH | Potential Hydrogen |
| Pup | Prokaryotic Ubiquitin-like Protein |
| PVDF | Polyvinylidene Difluoride |
| SAMPs | Small Archaeal Modifier Proteins |
| SDS | Sodium Dodecyl Sulfate |
| TBS | Tris-Buffered Saline |

# ABSTRACT

Suknaic, Stephen R. M.S., Purdue University, August 2013. Investigation of the Potential Bacterial Proteasome Homologue Anbu. Major Professor: Andrew Kusmierczyk.

Anbu is a bacterial protein with significant homology to the sub-units of the 20S proteasome and is predicted to be a novel bacterial proteasome. The goal of this project was to determine if the recombinant Anbu protein from *Pseudomonas aeruginosa* is a proteasome. Anbu from *P. aeruginosa* was successfully cloned, expressed and purified. In order to determine the catalytic activity of Anbu, the purified protein was tested with a variety of substrates and conditions. The targets analyzed included fluorescently-labeled substrates, denatured proteins, diubiquitin, and a peptide library in the hopes of obtaining a useful model substrate. Experiments were also conducted to determine what role Anbu has in the cell. Western analysis was performed on the cell lysate of wild type *P. aeruginosa* and insertional mutants to detect Anbu expression. The level of biofilm formation was compared between the wild type and mutants. Cultures were grown under stress conditions including the oxidative stress of diamide and the nitrosative stress of S-nitrosoglutathione. Growth rates were monitored in an attempt to detect a phenotypic difference between the wild type and the mutants lacking Anbu, HslV, and the other proteins of interest. While a substrate for Anbu has yet to be found, this protein was

found to assemble into a larger structure and *P. aeruginosa* lacking Anbu was sensitive to the oxidative stress of diamide and the nitrosative stress of S-nitrosoglutathione.

# CHAPTER 1: INTRODUCTION

## 1.1 The Proteasome

Proteasomes are large molecular machines responsible for a significant amount of protein degradation in cells and are found in all domains of life. They are responsible for degrading misfolded, damaged, and short-lived proteins in the cell and are necessary for cell viability (*1*) (*2*). Proteasomes are members of the Ntn-hydrolase (N-terminal nucleophile) superfamily of enzymes. This group of proteins is so named for the catalytic residue at the N-terminal position. These enzymes are capable of cleaving amide bonds and degrading proteins (*3*).

The 20S proteasome structure has four stacked rings, each comprised of seven distinct subunits. Two outer rings contain only the α-subunits, while the two inner rings contain the catalytically active β-subunits. One characteristic of the α-rings is the central pore which needs to be opened in order to allow for substrate entry into the catalytic sites of the inner chamber. A tightly regulated central pore is important, or non-specific protein degradation may occur within the cell (*4*). The pore can be opened by the action of ATPases which bind to the α-ring and open the central pore in an ATP-dependent manner. This action allows for a substrate to be threaded into the catalytic region of the core particle where they are degraded (*5*). The products from degradation are short peptide chains that can range in length (*6*).

## 1.2 The Eukaryotic Proteasome

One feature of Eukaryotic proteasomes is that each ring of the 20S core particle contains 7 unique subunits. The α-rings contain 7 distinct α-subunits, and the β-rings contain 7 unique β-subunits (*7*). Within the β-rings, the only subunits with catalytic activity are 1, 2, and 5 (*8*). The core particle in Eukaryotes can be capped on one or both ends by the 19S regulatory particle (RP) (*9*). Singly capped core particles have a size of 1.66 MDa while the doubly-capped proteasomes are 2.59 MDa (*10*).

Two structures comprise the 19S Regulatory Particle, the base and the lid. Traditional models of the 19S RP have portrayed these sections as two distinct regions. In these models, the base associates with the α-ring and the lid is in a distal position atop the base. Recent electron microscopy data shows that there is less of a defined separation as previously thought. Instead of being positioned on top of the 19S RP, the lid is oriented more so on the side and associates with both the base and the α-ring of the 20S core particle (*11*).

The AAA+ ATPase structure that binds the α-ring and is responsible for the unfolding of substrates is located in the base of the 19S RP. This hetero-hexamer is comprised of 6 Rpt subunits (Rpt1-6) and they are arranged in a spiral staircase orientation (*11*). When ATP binds to the AAA+ ATPase ring of the 19S RP, several important events occur. This action initiates the unfolding of a substrate, opens the central pore, and begins the translocation of the unfolded protein into the core particle for degradation (*12*) (*13*). In addition to the Rpt subunits, the base also contains Rpn1, 2, 10, and 13. The lid structure contains 9 non-ATPase Rpn subunits (*14*).

## 1.3 The Archaeal Proteasome

In addition to all Eukaryotes having a 20S proteasome, this compartmentalized structure is also found in all Archaea. However, there are some noteworthy differences between the two. While the α and β-rings of Eukaryotes contain seven unique α and β-subunits, the rings of Archaeal proteasomes typically contain only one or two unique α-subunits and one or two unique β-subunits. Additionally, the Archaeal proteasome is not capped on either end by the 19S Regulatory Particle like the Eukaryotic proteasome. Instead, the Archaeal 20S is capped by the proteasome-activating nucleotidase (PAN) (*15*). PAN has homology with the ATPases of Eukaryotes, and it similarly requires ATP for functionality. This structure is capable of refolding or unfolding other proteins (*16*). When PAN caps the proteasome, it increases the activity of the Archaeal 20S core particle (*15*).

## 1.4 The Bacterial Proteasome

While 20S proteasomes are found in all Eukaryotes and Archaea, they are only found in the Actinobacteria family of Prokaryotes. The current evolutionary model suggests that this family of bacteria acquired the 20S proteasome via horizontal transfer from Archaea (*17*). Similar to Archaea, these proteasomes contain only one or two variants of α-subunit and β-subunits (*18*). *Mycobacterium tuberculosis* contains an ATPase ring that associates with the 20S core particle called Mycobacterial proteasome ATPase (Mpa) (*19*).

## 1.5 Protein Modifications and Proteasome Degradation

In Eukaryotes, the small modifying protein ubiquitin plays a critical role in targeted protein degradation. Monoubiquitination occurs though the formation of isopeptide linkages with lysine residues of the target and the C-terminus of ubiquitin. Once monoubiquitination occurs, a second ubiquitin can attach in the same way to the first ubiquitin. This forms the beginning of a polyubiquitin chain (*20*). Proteins with regulatory functions such as DNA repair are typically modified via polyubiquitin chains on the K63 residue of ubiquitin. By contrast, a protein that is targeted for degradation, such as a misfolded or oxidatively damaged protein, is polyubiquitinated via the K48 residue of ubiquitin (*21*). This type of chain marks the protein for degradation by the proteasome (*22*). When a K48 polyubiquitinated protein encounters the proteasome, the chain is removed by the deubiquitinating enzyme Rpn11 to prevent the undesired degradation of ubiquitin (*23*) (*24*).

This process of targeted protein degradation is vital to a cell. Without ubiquitin, protein degradation by the proteasome would be reduced to a fraction of its normal levels (*25*). Ubiquitination and subsequent targeted protein degradation are also necessary for successful completion of mitosis. If the K48 residue of ubiquitin is mutated in yeast, the organism will enter a state of cell arrest in the midst of division (*26*).

Eukaryotic cells have ubiquitin as a means of modifying a protein post-translationally and Archaeal cells have a similar protein modification mechanism. Small Archaeal Modifier Proteins (SAMPs) have been identified that are ubiquitin-like and used in Archaeal cells to modify proteins. So far, the two proteins SAMP1 and SAMP2 have been identified as SAMPs. While these proteins have been characterized, their full

role in Archaea has yet to be determined (*27*). There are also enzymes that are capable of removing SAMPs from the protein to which they have been attached (*28*).

Like Eukaryotes and Archaea, Bacteria also have a post-translational modification process for proteins. For example, in *Mycobacterium tuberculosis* proteins are modified on lysine residues by a small, prokaryotic ubiquitin-like protein (Pup) (*29*). When proteins are modified with Pup, they are destined for degradation by the 20S proteasome (*30*). Given the functional similarity to ubiquitin, there are also noteworthy differences between the two proteins. When Pup encounters a proteasome, the N-terminus of Pup interacts with the Mpa-proteasome and is subsequently unfolded. The unfolded Pup is then fed through the central pore that leads into the catalytic chamber of the proteasome. As Pup is being unfolded and degraded, the protein that is still attached to Pup is also unfolded and fed into the proteasome. Both proteins are degraded as a result of this pathway. This process differs from the Ubiquitin-Proteasome pathway in Eukaryotes since Pup is degraded with each substrate while ubiquitin is removed prior to degradation and can then be reused (*31*).

## 1.6 The Compartmentalized Protease HslV

While the Actinobacteria are the only family that possesses the 20S proteasome, many other Bacteria have a different structure that contributes to the degradation of misfolded proteins. The heat-shock locus protein (HslV) is found in many bacteria that lack the proteasome. HslV assembles into a double-ring containing six subunits in each ring (*32*). This dodecamer is then capped on both ends by a hexameric ATPase ring of HslU proteins, forming the HslVU complex (*33*). The capping of HslV is important, for

the unregulated dodecamer has protease activity. However, this level of activity is substantially lower than when HslU is present (*34*). It has also been predicted that the HslV protein has a cation binding region near the catalytic site that may play a role in activity (*35*). The crystal structure of HslV and its similarities to the proteasome have led others to suggest that it is an evolutionarily distant relative of the 20S core particle (*36*). However, although much more widely distributed than 20S proteasomes, HslV is nevertheless not present in all bacterial lineages. This leads to the question of whether or not additional compartmentalized proteases exist in bacteria that have homology to the proteasome and have similar functions.

## 1.7 The Discovery of Anbu

Bioinformatic analyses have been carried out to determine if there are additional members of the proteasome family found in bacteria. A total of 238 bacterial genomes were screened to identify unknown proteins that may have homology to the α and β subunits of the 20S proteasome. Two novel proteins were found in this search. The first protein was given the name 'Anbu' while the second was named '*B-proteobacteria* Proteasome Homologue' (BPH) since it was only identified in this family of bacteria (*37*).

Anbu was identified in 24 genomes and was commonly inherited with 3 other genes in a predicted operon. The distribution of Anbu also had a much more interesting pattern than BPH. The organisms with Anbu were sparse but spread across several families of bacteria. This implies that Anbu may have been more commonly found and has since been lost over evolutionary time. However, this plausible explanation has yet to

be proven. Additionally, Anbu, BPH, and HslV were found in combinations with one another but never in the same genome with the 20S proteasome. This distribution pattern implies that HslV, Anbu, and BPH have unique functionality amongst one another and redundant activity with the 20S proteasome. Using sequence alignments and structural modeling, the authors made several predictions about Anbu and how this protein is related to the 20S proteasome (*37*). The following observations form the foundation of this project.

Sequence identity between Anbu from *Rhodopseudomonas palustris* was found to have an 18% identity at the amino acid level with the Archaeal 20S proteasome. Structural comparisons were conducted between HslV, Anbu, BPH, and the 20S β-subunit from *Thermoplasma acidophilum* predicts that there is a great deal of similarity between Anbu, the α-subunit, and the β-subunit of the 20S proteasome. Also, Anbu has a highly conserved Thr1 residue that could be responsible for a nucleophilic attack on a substrate. These structural similarities are put forth as evidence that the Anbu may be a novel proteasome (*37*).

The discovery of Anbu calls into question the current thinking about the evolution of proteasomes. The previous model of proteasome evolution suggests that the 20S proteasome is distantly related to the heat-shock locus protein, HslV (*38*). Due to the amount of predicted structural similarity between Anbu, the α-subunit, and the β-subunit, it has been proposed that 20S proteasomes may be more closely related to Anbu than HslV. Given that HslV forms hexameric rings, should Anbu assemble into a heptameric ring, it would yield further credibility to this hypothesis.

There is also another prediction as to the function of Anbu and its associated proteins. Of the three coding regions that are commonly inherited with Anbu, 2 are currently without a predicted function while the remaining protein resembles a transglutaminase [Figure 19]. It has been postulated that Anbu and this transglutaminase may function as a part of a peptide cleaving system in lieu of a proteasome. These predictions are based on the theoretical transglutaminase neighboring Anbu and the lack of a AAA+ ATPase near these coding regions (*39*).

## 1.8 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative bacterium commonly found in the environment. While this organism is normally non-pathogenic to healthy adults, the immunocompromised are susceptible to opportunistic infection. One of the well-studied forms of opportunistic *Pseudomonas* infections occurs in the lungs of Cystic Fibrosis patients. Part of the difficulty in treating infections relates to this organism being capable of producing a biofilm that makes it highly resilient to environmental stressors (*40*). A biofilm is a complex extracellular matrix excreted by a community of bacteria that are affixed to a surface (*41*). The biofilm produced by *P. aeruginosa* strain PA14 contains DNA and lipopolysaccharides (LPS) (*42*). The increased pathogenicity of a biofilm infection is caused by the organisms ability to tolerate environmental stressors and gain antibiotic resistance over time (*43*). Another factor that has been implicated in the antibiotic resistance and ability to survive harsh environments is the large genome of *P. aeruginosa*. In the year 2000, it was the largest bacterial genome to be sequenced having 6.3 million base pairs (*44*).

Since *P. aeruginosa* is not a member of the Actinobacteria, it does not have a 20S proteasome. However, it does have both HslV and Anbu proteins. To date, there has only been one publication showing an experimental finding related to Anbu and *Pseudomonas*. Expression of Anbu mRNA, but not of HslV mRNA, has been shown to increase during nitrogen starvation conditions in *Pseudomonas pudita*. The results from this experiment predict that Anbu plays a role in alleviating stress under nitrogen starvation conditions (*45*). Using strain PA14 (*46*) and insertional mutants derived from it will allow in vivo experiments involving Anbu, HslV, and other proteins of interest to be performed. Predicted structural similarities between Anbu and the 20S proteasome may allow for Anbu inhibition using known proteasome inhibitors, or their derivatives. New drugs may sensitize *P. aeruginosa* and lead to new therapeutic treatments for infections akin to how proteasome inhibitors may be useful against cancer (*47*).

## 1.9 Project Goals and Objectives

Despite the structural and evolutionary implications surrounding the Anbu protein, there are no publications describing any experimental analysis of this protein. Also, further understanding of this protein and its role in Bacteria may lead to potential treatments for infection. It is the goal of this project to conduct a full investigation of the Anbu protein using *P. aeruginosa* as the primary model organism, wherein Anbu is hypothesized to be a novel bacterial proteasome. To test the validity of this hypothesis, this thesis will focus on three inter-related aims:

1. Clone and characterize the Anbu protein in vitro.
2. Investigate the peptidase/protease activity of Anbu.
3. Characterize the in vivo role of the Anbu protein in *P. aeruginosa.*

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Cloning Sequences

Primers were designed to amplify Anbu and the coding regions of the other 3 genes in the predicted neighboring operon from PA14 with flanking NdeI and XhoI cut sites [Table 1]. Two sets of reverse primers were designed. One set would produce the authentic C-terminus of the proteins of interest; the other set would produce a C-terminal glycine-serine linker followed by a hexahistidine tag. PCR reactions contained 5 % DMSO (Sigma) and PA14 lysate that had been boiled as template (*48*). The reaction products were analyzed by electrophoresis in a 0.8 % agarose gel at 90 V until the dye front reached the bottom of the gel. Bands of the expected size were cut from the gel and extracted using Qiagen Gel Extraction kit and protocol. Purified extracts were then ligated into the pCR2.1 TOPO vector (Life Technologies) and transformed into the TOP 10 F' E. coli strain (Invitrogen). Individual colonies were plucked and grown in 5 mL LB with 50 ug/mL kanamycin (LB-kan) and shaking overnight at 37 °C. Plasmids were isolated from these cultures following Qiagen protocol followed by a double restriction digest with NdeI and XhoI (New England Biolabs). Digest products were then analyzed by electrophoresis in an 0.8% agarose gel run at 90 V. Mini-preps with correct digest products were sent out for sequencing by a commercial provider (ACGT, Inc). Glycerol stocks of sequence-verified plasmid constructs were saved. NdeI and XhoI fragments of

the correct gene sequences were subcloned into pET42 expression vector that had been successfully digested with both of these enzymes.

In addition, a synthetic plasmid was ordered from Genscript containing the coding region of Anbu from *Klebsiella oxytoca* to serve as an additional protein for experimental analysis. This sequence was also designed to encode a C-terminal hexahistidine tag and flanking NdeI/SacI restriction digest sites. This plasmid was transformed into TOP 10 F' *Escherichia coli* strain to prepare a stock culture and begin subcloning into pET42 expression vector. Preparation of this construct was performed in the same fashion as previously described, save for the use of NdeI/SacI restriction enzymes instead of the NdeI/XhoI. This version of Anbu has been annotated as 'KAnbuH' to distinguish it from the *P. aeruginosa* counterpart.

## 2.2 Induction in BL21 cells

Mini-preps of the pET42 expression vector constructs were transformed into the BL21 expression strain (Novagen) and grown overnight on LB plates with 50 µg/mL kanamycin. Individual colonies removed and grown in 3 mL LB-kan for 4 hours at 37 °C with shaking. Cultures were then supplemented with 3 mL LB-kan and IPTG (US Biological) to a final concentration of 1 mM and allowed to incubate at 37 °C with shaking for 3 hours. They were then pelleted by centrifugation at 13,000 × g for 1 minute and stored at - 80 °C for future analysis. This protocol was always performed using freshly transformed BL21 cells from plates that were not more than 1 day old.

## 2.3 Fractionation Analysis of Proteins of Interest

Pellets were lysed by resuspending in a buffer comprised of 50 mM Hepes-NaOH pH 1.5, 150 mM NaCl, 5 mM $MgCl_2$, 40 mM Imidazole (Buffer A) with the additions and final concentrations of 0.1% Triton X-100, 300 ug/mL lysozyme, 10 ug/mL DNase I, and 2 mM pefabloc. The suspension was then agitated for 30 minutes at 30 °C. Fractions were taken from this lysate that represent the total fraction, and another fraction that will be spun at 13,000 × g for 10 minutes. The supernatant and pellet were saved separately as the soluble and insoluble fractions, respectively, for subsequent analysis. The remainder of the soluble fraction with the his-tagged proteins was purified using GE Spintrap Ni-NTA columns (GE Healthcare) according to manufacturer's protocol. Soluble cell lysate containing the his-tagged proteins was loaded onto the columns, spun at 100 × g for 1 minute, and a fraction of this 'flow though' was saved for subsequent analysis. Columns were rinsed 3 times with 600 µL of Buffer A to remove as much residual protein as possible. Elution was carried out with 2 applications of 200 µL of Buffer B comprised of 50 mM Hepes-NaOH pH 7.5, 500 mM NaCl, 5mM $MgCl_2$ and 500 mM Imidazole. Centrifugation then occurred at 100 × g for 1 minute per wash. Eluate was either analyzed or preserved by adding 75% glycerol (Sigma) to a final concentration of 15% glycerol and then stored at -80 °C. The total, pellet, soluble, flow through, and eluate fractions were analyzed by 10%, 12%, or 15% SDS-PAGE.

To attempt to minimize proteolytic activity on the Anbu protein, 10 µL/mL of HALT protease inhibitor cocktail (Thermo Scientific) was added to the cell lysis buffer. EDTA was also used at a final concentration of 1 mM. The Ni-NTA columns were not used to purify the his-tagged proteins from the soluble lysate with the addition of EDTA.

In place of purified protein, the soluble fraction obtained after the lysis step and centrifugation was analyzed.

## 2.4 SDS-PAGE Analysis of Protein Fractions

SDS-PAGE gels were prepared with a stacking gel of 4% polyacrylamide and a resolving gel of 10%, 12%, or 15% depending on the proteins to be analyzed. Protein samples were denatured by adding an appropriate volume of 5X Laemmli Sample Buffer, followed by boiling on a 95 °C hot block for 3-5 minutes. Electrophoresis occurred first at 80 V to allow the protein to enter the stacking gel. As the proteins entered the resolving gel, the voltage was increased to 120 V. For analysis with large gels, electrophoresis occurred first at 120 V to allow the protein to enter the stacking gel. As the proteins entered the resolving fraction, the voltage was increased to 180 V. The gels were allowed to run until the bromophenol blue dye front had left the gel. Gels were then washed 3 times in deionized water for 10 minutes per wash. After the final wash, the water was decanted and gels were stained with Gelcode blue (Thermo Scientific). The gel was allowed to agitate on a rocking platform until bands could be seen. To obtain better resolution of bands, a volume of water was added to sufficiently dilute the stain and the gel was stained overnight with rocking. The gels were scanned for a permanent record on the Perfection V700 (EPSON) and then dried.

## 2.5 Western Blot Analysis

Samples to be analyzed were prepared by first performing SDS-PAGE as explained in 2.4. Gels were then equilibrated in a volume of transfer buffer adequate to

soak the gel for 15 minutes. Filter paper was cut to the size of the gels and also soaked in transfer buffer. PVDF membrane Immobilon (Millipore) was first cut to size, wetted with methanol, and then allowed to soak in transfer buffer for 15 minutes. Once all components had been sufficiently equilibrated in transfer buffer, the transfer sandwich was assembled in the Trans-Blot D Semi-dry Transfer Cell blotting apparatus (Biorad). After each layer was applied a test tube was used to roll out any air bubbles. The apparatus was then assembled and the gel transferred at 15 V for 25 minutes. Once the transfer was complete, the PVDF membranes were placed into a Blocking Buffer consisting of Tris-Buffered Saline (TBS) and 5% non-fat dry milk. The membranes were allowed to block overnight while rocking. Blocking buffer was then decanted, and the membranes were exposed to a solution of the primary antibody in blocking buffer for 90 minutes while rocking. The primary antibody solution was then removed and stored at -80 °C for repeated use. Membranes were then washed 3 × 5 minutes with a buffer containing 50 mM Tris-Base, 150 mM NaCl and a pH of 7.5 (TBS) and containing 1% Tween 20 (TBS/Tween). Blots were then exposed to a solution of the appropriate secondary antibody in TBS/Tween and allowed to incubate at room temperature for 60 minutes with rocking. The secondary antibody solution was then decanted, and the blot was washed 3 × 5 minutes with the TBS/Tween solution. Blots were removed from the wash solution and placed in a plastic page holder. Blots were developed using enhanced chemiluminescence reagent Pierce ECL Blotting Substrate (Thermo Scientific) and exposed to CL-Xposure film (Thermo Scientific). Common times for exposure were 30 seconds, 5 minutes, or overnight. The resulting developed film was then scanned for permanent record and editing.

## 2.6 Native PAGE Analysis

The reagents used for this analysis do not contain agents such as SDS or DTT. These reagents denature proteins so they can be analyzed as in 2.4. The native PAGE method analyzes proteins in non-denatured, or native, state. Since the samples that were analyzed have a much larger predicted size when compared to their denatured counterparts, native PAGE was performed with polyacrylamide gels comprised of a 4% stacking gel and a 5% resolving gel. Protein samples to be analyzed were prepared with an appropriate volume of 5x non-denaturing loading buffer containing 50% glycerol and 300 mM Tris-HCl pH 6.8 and bromophenol blue dye. For analysis on mini gels, electrophoresis occurred first at 50 V to allow the protein to enter the stacking gel. As the proteins entered the resolving fraction, the voltage was increased to 60 V. For analysis with large gels, samples were then loaded into the wells of the gel in the assembled rig. Electrophoresis occurred first at 50 V to allow the protein to enter the stacking gel. As the proteins entered the resolving fraction, the voltage was increased to 65 V. The small gels were allowed to run until the bromophenol blue dye front had left the gel, while the large gels were run overnight. Following electrophoresis, gels were rinsed in deionized water and stained with GelCode Blue until bands were visible. To obtain better resolution of bands, a volume of water was added to sufficiently dilute the stain and the gel was rocked overnight. The gels were scanned for a permanent record and then dried.

## 2.7 In Vitro Translation and Concentration

Pre-sterilized filtered pipet tips were used throughout this protocol to limit environmental contamination of the reaction. In vitro translation was carried out using the

EasyXpress kit (Qiagen) according to manufacturer's protocol. Briefly, reagents were kept on ice save for the RNAse free water, which was allowed to thaw at room temperature. The reaction was prepared in the *E. coli* Extract tube by adding 11 µL of RNAse free water, 1 µL of the pET42 plasmid containing the sequence to be translated, 20 µL of the EasyXpress Reaction Buffer, and 0.5 µL of 100 mM stock IPTG solution. The tubes were incubated at 37 °C for 1 hour to translate the desired protein. Proteins were then purified using Ni-NTA columns as in 2.3.

In order to obtain an elution fraction with a quantity of protein suitable for analysis, the eluted proteins were applied to either the Vivaspin (GE Healthcare) or Amicon 0.5 (Millipore) concentrators and centrifuged at 14,000 × g until the excess buffer had passed though the membrane. The concentrated protein and a fraction of the flow though were removed and stored for analysis. To investigate the possible adsorption of protein by the membrane, a 50 µL volume of running buffer with SDS was carefully pipetted across the membrane as a wash step. The wash solution was then collected and stored for analysis. Fractions of the initial load, flow though, concentrated protein, and wash solution were analyzed by SDS-PAGE as in 2.4.

## 2.8 In-Gel Peptidase Assay

Samples were analyzed by native PAGE as described in 2.6 with multiple replicates of the same sample sets. After the gels were removed from the casting plates, they were carefully cut with a razor to separate each replicate. Gels were then soaked in 10 mL of a substrate solution containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10% glycerol, and 10 mM of a substrate (Ex. Suc-LLVY-AMC) from the Proteasome

Substrate Pack (Enzo). Optional additions to this substrate solution were 1 mM ATP and 0.06% SDS. Due to the small volume of substrate solution used, the gels were incubated in petri dishes at 37 °C for 30 minutes to allow for better exposure and increased activity (*49*). After incubation, the gels were carefully removed from their dishes and placed on a UV transilluminator for visualization. Photographs were taken of gels that had visible banding patterns. The gels were then briefly washed in deionized water prior to staining with GelCode Blue as in 2.6. Photographs of the stained gels were taken to compare the fluorescent banding patterns to the stained proteins.

## 2.9 Diubiquitin Digest

Elution fractions of the proteins to be analyzed were obtained following the protocol in 2.3. Samples were prepared in duplicate and 1.25 µg of either K48 or K63 linked diubiquitin (Enzo) was added to each aliquot of proteins and mixed. Samples were incubated at 37 °C and aliquots were removed at time points 0, 1 hour, and overnight. Samples were removed and analyzed by SDS-PAGE as in 2.4.

## 2.10 Protease Activity Assay

Proteins to be analyzed were purified as in 2.3. The assay was carried out using the QuantiCleave kit (Thermo Scientific) as per manufacturer's protocol and follows the degradation of succinylated casein substrate. A 96-well microtiter plate was prepped by pipetting 100 µL of succinylated Casein Solution in triplicate for each protein to be analyzed. One set of wells served as a control containing 100 µL of Borate Assay Buffer and no casein. A volume of 50 µL of the purified protein was added to each well. The

positive control protein solution was a 1:50 dilution of trypsin in Buffer B. The plate was covered and allowed to incubate at 37 °C for 30 minutes. After incubation, 50 µL of the TNBSA Working Solution was added to all of the wells to develop color. The plate was covered and incubated overnight at 37 °C. Absorbance was then measured on the Spectramax M2 spectrophotometer (Analytical Technologies) at 450 nm.

## 2.11 Small Protein Degradation Assay

Three proteins, α-lactalbumin, β-casein, and lysozyme (all from Sigma) were prepared in 100 mg/mL stocks and were thawed prior to use. The proteins were denatured in a solution containing 89 µL of 9 M urea, 1 µL 2 M DTT, and 10 µL of protein stock. All aliquots were incubated at 30 °C for 30 minutes. Proteins to be analyzed were purified as in 2.3. A buffer solution containing 50 mM Hepes-NaOH pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$ (HNM buffer) was prepared that contained 2 mM DTT. Analysis began by adding 10 µL of the protein of interest to 89 µL of the DDT supplemented HMN buffer and adding 1 µL of the denatured protein. At time 0, 1 hour and overnight, aliquots were removed and denatured with 5x Laemlli buffer and boiled for 3 minutes. Samples were analyzed on 15% SDS-PAGE as in 2.4.

## 2.12 Peptide Library Screen

To investigate the potential peptidase activity of Anbu, a peptide library in 96-well format (Pepset) was purchased from Mimotopes. A Pepset plate was prepared by first adding 5 µL of a 50 % (v/v) acetonitrile solution in water to each well. A volume of 45 µL of assay buffer containing 50 mM Tris, 150 mM NaCl, 0.2% PEG was then added

to each well. Baseline levels of fluorescence were then acquired on a Spectramax plate reader. The excitation wavelength was 320 nm and emission wavelength was 420 nm. Proteins to be analyzed were purified as in 2.3 and were diluted 1:10 in the assay buffer. A volume of 50 µL of the protein solution was then added to each well and analyzed by the Spectramax plate reader every 2 minutes for 90 minutes. After the last reading, the plate was removed and 8 µL of another purified protein was overlaid to each well, and the plate was read once more following the same parameters. This was done to maximize the use of the library, especially using wells where no signal was obtained in the first incubation. Once the second analysis was complete, the data was saved and the plate was stored at - 80 °C.

## 2.13 Biofilm Analysis

Cultures of *P. aeruginosa* PA14 wild type strain, as well as strains containing transposon-disrupted open reading frames encoding various proteins of interest were obtained (kindly provided by Dr. Gregory Anderson) and grown overnight in 5 mL cultures of LB with 15 µg/mL tetracycline. Optical density measurements ($OD_{600}$) were taken at 600 nm, and the cultures were diluted 1:100 in fresh LB. Prior to dispensing a Seroculture 96-well plate with rounded bottoms (Costar) was carefully cut in half. Aliquots of 100 µL were then pipetted in replicates into each of the two halves. The plate was covered, carefully wrapped with a moist paper towel, placed in a plastic bag, and incubated at 37 °C without shaking. After 24 hours, one half of the plate was removed, while the other half was allowed to incubate for a full 48 hours. Cultures were decanted and the plate was gently washed in deionized water. The water was then decanted, and

the plate was blotted with paper towels to remove any excess water. Each well was then filled with 125 µL of a 0.1% (w/v) crystal violet solution. This stain was allowed to set for 10 minutes before the crystal violet was decanted. The stained plate was washed in a bath of deionized water and decanted before a final wash and decanting step was performed. The washed plate was then blotted with a paper towel to remove any excess water, wrapped in a paper towel, labeled and allowed to dry at room temperature. When the plate had dried, the stained biofilm was solubilized with the addition of 150 µL of 30% (v/v) acetic acid to each well. After incubating for 10 minutes, a volume of 125 µL was removed from each well and pipetted into a flat bottom 96-well microtiter plate (*50*). Absorbance at 600 nm was measured using the Spectramax plate reader.

## 2.14 Growth Curve Analysis

Cultures of the *P. aeruginosa* PA14 wild type strain and transposon-disrupted mutant strains of interest were grown overnight in 5 mL cultures of LB with tetracycline. Following measurements of $OD_{600}$, the cultures were diluted to an optical density of 0.3. An aliquot of each culture was pipetted into a microcentrifuge tube and centrifuged at 13,000 × g for one minute to pellet the cells. The supernatant was removed and the cells were suspended in fresh LB. A volume of 50 µL of the resuspended culture was added to each well of a 96-well microtiter plate. Various solutions of stress-inducing molecules, prepared in LB, were then added in an array of final concentrations to determine the level of stress each imposed on the organism. The final volume of all wells was 100 µL, and all wells were prepared in duplicate to fill the entire plate. After the lid was placed on the plate, a layer of Parafilm was wrapped around the outside edge to minimize moisture loss

over time. The plate was analyzed at 37 °C by measuring $OD_{600}$ in the Spectramax plate reader. An initial reading was taken and subsequent readings were taken every 20 minutes, immediately after a 3 second shaking period, for a total of 12 hours.

# CHAPTER 3: RESULTS AND CONCLUSIONS

## 3.1 Anbu Expression

DNA fragments coding for untagged and C-terminally hexahistidine tagged (his-tagged) Anbu from *P. aeruginosa* PA14 were amplified from bacterial genomic DNA and subcloned into the pET42 expression vector. The successfully completed constructs were transformed into *E. coli* BL21 expression cells. Protein expression was induced with IPTG and clarified lysates from the induced cell pellets, as well as no-plasmid control pellets, were fractionated into total (T), pellet (P), and soluble (S) fractions. The soluble fractions were subsequently applied to Ni-NTA columns and the flow though (FT) was collected. Bound proteins were eluted (E) with imidazole containing buffers. When these fractions were analyzed by SDS-PAGE for the non-his tagged protein, a prominent band was observed in the T, P, S, and FT lanes, running just below the 25 kDa marker. A strong band was not detected in the E fraction [Figure 1]. The size of this protein is close to the predicted molecular weight of Anbu, which is 26.3 kDa. This band was not present in the control BL21 fractions, which indicates that the protein was produced during the induction protocol.

The analysis of the T, P, S, FT, and E fractions was also performed to identify the his-tagged version of the Anbu protein. When these fractions were analyzed by SDS-PAGE, a prominent band was observed in the T, P, S, and E lane, running slightly above the 25 kDa marker [Figure 2]. Due to the addition of the histidine tag, this form of the

protein would be expected to run slightly slower on SDS-PAGE. Additionally, the eluted protein appeared to be present in the form of a doublet (see below).

The analysis of the T, P, S, FT, and E fractions was also performed to identify the his-tagged version of the *K. oxytoca* Anbu (KAnbuH) protein. When these fractions were analyzed by SDS-PAGE, a prominent band was observed in the T, P, S, and E fractions, running above the 25 kDa marker [Figure 3]. The slower migration is expected since the *K. oxytoca* Anbu has a predicted mass of 28.6 kDa. By contrast, this protein appears to be running as a single band, whereas a doublet was present in the *P. aeruginosa* Anbu.

## 3.2 Native PAGE Analysis

Having successfully purified the recombinant protein, the next point to consider was that of assembly. In order to determine if the protein assembled into species larger than a monomer, a sample of the his-tagged Anbu was analyzed by Native PAGE [Figure 4]. In addition to commercially available protein standards, two additional samples were purified and analyzed alongside Anbu to provide a size reference: Archaeal α-subunits that form single (~200 kDa) and double (~400 kDa) homoheptameric rings, as well as complete Archaeal 20S proteasomes (~700 kDa). Under non-denaturing conditions, the his-tagged Anbu protein from *P. aeruginosa* was running at a comparable position to the single α-ring of the Archaeal proteasome and the 140 kDa size standard. Additionally, the his-tagged Anbu protein from *K. oxytoca* was running at a comparable position to the double α-ring. These findings are noteworthy, since the non-denatured proteins are running at a significantly larger size than the denatured protein. It indicates that the recombinant Anbu protein spontaneously assembles into a structure several times larger

than individual monomers. It is tempting to speculate that, compared to the mobilities of the single α-ring (~200 kDa) and the molecular weight standard (~232 kDa), the his-tagged Anbu may be forming a heptameric or octameric complex. However, since this is a non-gradient Native PAGE, it is not possible to infer exact size information or assembly configuration from the position of the standards.

## 3.3 Resolving the Doublet

Preliminary analysis of his-tagged Anbu suggested the purified protein may be migrating as a doublet on SDS-PAGE [Figure 2]. In order to better visualize the two different sized species of Anbu that are forming the doublet, the eluate was analyzed by SDS-PAGE on a large gel [Figure 5]. This analysis would also eliminate the possibility of the banding pattern occurring due to a gel artifact by resolving the doublet into two distinct bands. If two different sized proteins are produced, then the difference in migration distance will increase with the size of the gel. The stained gel shows the purified his-tagged Anbu separating into two bands that are both close in size and intensity. In Figure 5, the two bands appeared in comparable intensity. In subsequent purification attempts, the ratio of the bands varied (data not shown) such that the bottom band was more intense than the top. However, regardless of the ratio, this finding confirmed that there were in fact two different sized Anbu species that are eluted off the Ni-NTA columns.

This doublet banding pattern, especially the varying intensity ratio of the bands, was disconcerting as it might be the result of endogenous *E. coli* proteases cleaving residues off the Anbu protein during the lysis step of purification. The N-terminus of

Anbu contains the putative catalytic threonine residue (Thr1), which becomes exposed following the removal of the initiator methionine. Should this residue be proteolytically removed during purification it would result in an inactive form of the protein, thus rendering further substrate analyses fruitless. Therefore, subsequent investigation involved attempts to eliminate the formation of the different sized bands.

## 3.4 EDTA and HALT Additions

The first attempts to eliminate the formation of the doublet included the addition of EDTA or a mix of commercially available protease inhibitors (HALT Protease Inhibitor Cocktail) to the lysis step of protein purification. The lysis buffer already contains the serine-protease inhibitor Pefabloc. However, these agents would further reduce the activity of endogenous *E. coli* proteases and the intent of their additions was to reduce the doublet to a single band. Samples of traditionally purified his-tagged Anbu with and without HALT supplementation and soluble lysate of Anbu-expressing *E. coli* treated with EDTA were prepared simultaneously and analyzed by large SDS-PAGE to determine what effects these additions had on doublet formation [Figure 6]. The banding pattern in the samples with no additions was a doublet with bands of similar intensity. Samples that contained the additional HALT also contained a doublet with comparable bands. When EDTA was added to the lysis, the lower band was much more prominent.

It follows that supplementing the lysis buffer with HALT was not capable of reducing the doublet to a single band. Also, the addition of EDTA resulted in an increase in the size of the lower band of the doublet. This increase could also have been the result of an active *E. coli* protease still present in the soluble lysate acting on the his-tagged

Anbu protein. Due to their unsuccessful elimination of the doublet, these two supplements were not used in subsequent purifications or experiments.

### 3.5 In Vitro Translation and Concentration

The second attempt to eliminate the doublet involved the production of Anbu in an in vitro translation system. This technique of protein production contained the purified translation machinery from *E. coli* and would allow for expression in an environment with as few endogenous proteases as possible. When the purified protein was analyzed by SDS-PAGE, the doublet had been almost completely reduced to a 'top-heavy' form where the lower band was barely visible [Figure 7]. However, the use of this expression method was not without flaws. The volume of protein produced was at an insufficient concentration to perform further analyses thus, the fractions would have to be concentrated.

Elution fractions concentrated and analyzed by SDS-PAGE yielded an unexpected result. Only a faint band could be detected whereas a several-fold increase in concentration was expected. To determine where the majority of the protein had relocated, the concentration steps were repeated and additional samples were analyzed. A fraction of the flow though was analyzed, in the event that the protein had somehow passed though the concentrator. Furthermore, to determine if the protein had aggregated on the membrane, the surface was gently washed with 1x Laemmli Running Buffer with SDS and also analyzed by SDS-PAGE [Figure 8].

The stained gel shows that, as expected, no protein was present in the flow though lane. As had previously been observed, very little His-tagged Anbu was recovered from

the fraction that should have contained the concentrated protein. The fraction that has the ideal banding pattern was the denaturing wash of the concentrator membranes. This lane contained a very strong band running at the expected size of his-tagged Anbu and was noticeably darker than the samples that were purified but not concentrated. These results indicated that the majority of the protein added to the concentrator was adsorbed to the membrane and was not successfully concentrated. A concentrator from a different company was employed, which contained a membrane of different composition, but the result was the same (not shown). Adsorbtion of protein to membrane can be indicative of an improperly folded protein, suggesting that the Anbu protein produced via in vitro translation may not be native. Therefore, due to the insufficient amount of protein produced from this analysis, and the complications involved in protein concentration, in vitro translation was not used for protein expression in further experiments.

### 3.6 Doublet Analysis by Western Blot

Since the doublet phenomenon could not be eliminated, the next course of action was to determine if proteolysis was occurring at the C-terminus or N-terminus of the his-tagged Anbu protein. Reasoning that C-terminal cleavage would preserve the essential N-terminal Threonine and could still result in an active enzyme, western analysis was performed to determine the status of the C-terminal his-tag. Samples of the his-tagged Anbu were analyzed by Western blot and probed to detect the his-tag. It was observed that only a single band was present in the his-tagged Anbu lanes [Figure 9]. Hence, only one of the bands in the doublet is tagged while the other is not. A subsequent Western blot was performed in a similar manner, however the antibody chosen was a polyclonal

rabbit antibody raised against Anbu. When visualized, the familiar doublet was present in the Anbu lane and had been carried over into other lanes [Figure 10]. Taken together, the data suggest that the doublet is in part a result of the loss of the C-terminal his-tag in some of the subunits although it cannot be excluded that one or perhaps a few additional C-terminal amino acids in Anbu itself are also lost. The results also implies that tagged and tagless Anbu subunits assemble together into a complex because they coelute from Ni-NTA; which is consistent with the presence of a larger structure as visualized on the native gel. Given that the doublet was not the result of N-terminal cleavage, thereby preserving the potentially N-terminal catalytic threonine, we proceeded to determine if Anbu possessed proteolytic activity.

## 3.7 Proteolytic Activity of Anbu

If Anbu is a novel type of bacterial proteasome, one would expect Anbu to possess the ability to degrade peptides and/or proteins. Several experiments were performed in an attempt to demonstrate activity in different ways.

### 3.7.1 In-Gel Peptidase Assay

Fluorogenic peptide substrates are frequently employed to demonstrate the proteolytic activity of 20S proteasomes. Thus, the first set of activity assays were performed with 5 different proteasome peptide substrates. In this assay, proteins are subjected to native PAGE and then gels were incubated with a solution containing one of the peptide substrates. If the electrophoresed protein possesses peptidase activity, this results in the release of the fluorescent AMC moiety from the peptide substrate and this

can be visualized on the UV transilluminator [Figure 11]. Activity was only observed for the Archaeal 20S core particle control. There was no activity observed in the lanes containing his-tagged Anbu for any of the model peptide substrates (only the gels for the Suc-LLVY-AMC peptide substrate are shown for simplicity).

It is possible that the in-gel assay lacks the sensitivity to detect the activity of Anbu. As a follow up, the substrates were analyzed using the fluorometer functionality of the Spectramax to monitor fluorescence overnight. Again, the only wells with activity were those containing the Archaeal proteasome [Figure 12]. These results reflect those found with the in gel assay for the Suc-LLVY-AMC.

### 3.7.2 Protease Activity Assay

In order to determine if the substrate for Anbu is a loosely folded or misfolded protein, the next experiment utilized succinylated casein as a potential substrate. Casein has a loosely folded orientation that is easily accessible to proteases and has been used as a model substrate for protein degradation. If the protein is cleaved during the analysis, a yellow color change occurs in which the intensity is proportional to the amount of protein degradation. The intensity of the yellow color relates directly to the amount of protein degradation. Proteins to be analyzed were purified and the reaction was allowed to proceed overnight in the microtiter plate. The following day, color change could be observed by inspection in the wells containing the positive control, trypsin and substrate. Readings from the spectrophotometer were taken and confirmed the color change [Figure 13]. Additionally, the Archaeal 20S core particle had higher absorbance than the negative

control of only α subunits. The readings for the two Anbu variants were at or below that of the control, indicating that they did not have quantifiable activity in this assay.

### 3.7.3 Small Protein Degradation Assay

Although casein lacks a defined structure, it is possible that it is not unfolded to a point where it is accessible to the catalytic sites of Anbu. To address this, three model substrate proteins were used as an alternative. Namely, α-lactalbumin, β-casein, and lysozyme were first chemically denatured in urea and DTT to ensure they would be unfolded prior to being added to Anbu. After incubation, the samples were analyzed by SDS-PAGE [Figure 14, 15, 16]. The only activity observed with the α-lactalbumin was in the lanes with the Archaeal 20S core particle. A dark band is present in the time zero lane, and this band becomes less prominent at the 1 hour and overnight time points. All of the samples of Archaeal α-subunits and his-tagged Anbu have comparable intensities across all time points, indicating the protein was not degraded. In both the β-casein and lysozyme analyses, the bands representing these proteins are of comparable intensity across the gel. This indicates that the proteins were not degraded, even in the presence of the Archaeal 20S core particle. One possible reason for this lack of activity is that the denatured proteins aggregated and were not able to enter the pore of the 20S proteasome.

### 3.7.4 Peptide Library Screen

With the fluorogenic substrates used in 3.7.1, cleavage must occur immediately prior to the AMC molecule in order for fluorescence to be detected. This condition is limiting to peptidase activity that requires residues both before and after the target site.

As another attempt at finding a substrate, a peptide array was analyzed containing substrates of a different configuration. Each of the peptide substrates contained in this array contained a fluorogenic reporter and a quencher linked by the peptide sequence (*51*). When a peptidase cleaves any point in the peptide chain, the quencher and reporter are separated. The subsequent increase in fluorescence can then be quantified. These substrates differ from those used in 3.7.1 in that cleavage can occur at any point in the peptide chain and result in fluorescence. As of this writing, four of six plates have been analyzed with both varieties of Anbu and no activity has been detected.

### 3.7.5 Diubiquitin Digest

The next assay was designed to determine if the his-tagged Anbu protein had isopeptidase activity as opposed to peptidase activity. Isopeptide linkages are not found in the backbone of a peptide chain but can nevertheless link two proteins together. For example, these bonds can form between the side chain amine of a lysine residue and a main chain, or side-chain, carboxyl group. The bond that links multiple ubiquitins together is an isopeptide linkage. If Anbu has activity similar to isopeptidases, then diubiquitin may be a useful model substrate for catalysis. Two forms of diubiquitin were tested to determine if they would be a useful substrate for Anbu. K48 and K63 linked diubiquitin were incubated with Anbu with the intent of hydrolyzing either of the two isopeptide bonds and forming monoubiquitin. Proteins for analysis were purified and incubated with either form of diubiquitin overnight. The following day, aliquots of each sample were analyzed by SDS-PAGE to determine if hydrolysis had occurred [Figure 17 and 18]. In both the K48 and K63 samples, no obvious degradation of diubiquitin was

observed and no monoubiquitin was observed. It should be noted that there was no positive control isopeptidase present in the lab for these experiments. This experiment will be revisited once a known isopeptidase has been obtained. As such, it is possible that activity was not observed due to sub-optimal conditions for cleaving the isopeptide bond.

## 3.8. Phenotype Determination

If Anbu is a novel type of bacterial proteasome, it would be expected that Anbu has an important role in cell viability. In an attempt to determine what role Anbu has in *P. aeruginosa*, wild type strain PA14 and mutants containing transposon disruptions in the genes of interest from the Anbu operon (kindly provided by Dr. Gregory Anderson) were grown in culture under different conditions and phenotypic observations were made. The insertional mutations of interest are annotated as m1730, m1731, m1732, mAnbu, and mHslV for transposon disruptions in the respective genes and WT for wild type PA14 [Figure 19].

### 3.8.1 Mutant Westerns

In order to help analyze the function of Anbu and the 3 unknown proteins in the associated operon, rabbit polyclonal antibodies were raised against all four proteins and required validation prior to use. Western blot analysis was performed on whole cell lysate samples of the *P. aeruginosa* PA14 wild type strain and transposon-disrupted mutant strains for each of the four proteins of interest. BL21 cell lysates containing the recombinant protein served as a positive control for antibody detection.

Of the resulting banding patterns from this analysis, the most informative was the anti-Anbu blot [Figure 20]. The polyclonal anti-Anbu antibody was able to detect the recombinant protein as an intense signal near the 25 kDa marker. While there were bands present in all the lanes containing *P. aeruginosa* cell lysates, there was an intense band at the same size as the recombinant proteins present in all lanes save for the Anbu mutant. This was an important finding for a number of reasons. First, it validated the anti-Anbu antibody in its ability to detect the genuine protein in its host organism. Second, it also validated the utility of the Anbu mutant strain, which was used in the analyses described below. Furthermore, it indicated that the Anbu protein was present under normal growth conditions in *P. aeruginosa*.

While the anti-Anbu blots yielded interesting findings, the other blots were less insightful. Although the other 3 polyclonal antibodies were able to detect the recombinant protein (not shown), no unambiguous banding patterns indicative of the presence of these proteins were visualized in the wild type or mutant strains analyzed. Modifications to the probing protocol, such as increasing the concentration of primary antibody used, were not successful at visualizing the proteins (data not shown).

### 3.8.2 Biofilm Assay

The formation of biofilm in *P. aeruginosa* is an important factor in infections. In order to determine if the Anbu protein has a role in this aspect of pathogenesis, biofilm growth was one phenotypic assay performed on the insertion mutations of PA14. All wells had sufficient growth prior to staining. Analysis of the biofilm stain by spectrophotometry showed comparable results for all of the wells at 24 hours [Figure 21].

At the 48 hour time point, readings were more varied, resulting in higher standard deviation. This is unsurprising, since the cells will become stressed after this amount of time. At both of these time points, the level of biofilm formation is comparable between the mutants and wild type PA14.

### 3.8.3 PA14 Mutant Growth Curves

Another approach in determining a cellular role for Anbu involved exposing the PA14 mutants to different stressful growth conditions. Stress conditions were selected with a preference for those that may induce stress via the misfolding of proteins. Preliminary experiments contained factors known to stress *P. aeruginosa* such as $H_2O_2$ (*52*) (*53*) (*54*), $CuCl_2$ (*55*), azetidine 2-carboxylic acid (AZC), diamide (*56*), S-nitrosoglutathione (*57*), and nitrogen starving conditions (*58*) (*59*).

Two of the growth conditions that seemed the most promising were caused by the oxidative stress of diamide and the nitrosative stress of S-nitrosoglutathione. When grown in just LB, the mutants and WT PA14 are comparable in growth rates [Figure 22, 25]. At 10 mM diamide, all of the mutants grew at a slower rate when compared to wild type, and the Anbu and HslV mutants showed the most sensitivity to this stress [Figure 23]. When exposed to the highest tested concentration (35 mM) of diamide, the Anbu mutant growth curve showed no increase in absorbance over time compared to wild type [Figure 24]. When exposed to 10 mM of S-nitrosoglutathione, all of the mutants grew at a slower rate when compared to WT PA14 [Figure 26]. The Anbu and HslV mutant showed the most sensitivity to this stress when compared to the other mutants and WT.

These results indicate that the stress caused by these two additions had an effect on the growth of the mutant strains compared to WT.

# CHAPTER 4: DISCUSSION AND FUTURE EXPERIMENTS

## 4.1 Assembly

Given the results from the SDS-PAGE and native PAGE analysis it is evident that both the *P. aeruginosa* and *K. oxytoca* recombinant forms of Anbu assemble into a larger structure. It is peculiar that these two variants are running at different sizes under non-denaturing conditions. While the *P. aeruginosa* Anbu is at a position comparable to a single α-ring (est. 200 kDa), the *K. oxytoca* Anbu is running at a position near the double α-ring (est. 400 kDa).

Although these observations are taken from a non-gradient Native PAGE, they have significant implications. Generally speaking, the compartmentalized proteases are comprised of assembled rings containing six or seven subunits. Since the two forms of Anbu are running at positions comparable to known ringed configurations, it is very likely that they are assembling into higher order structures of at least several subunits, which may also be ring shaped. However, it is impossible to determine the subunit stoichiometry of these higher order complexes from native PAGE analysis alone as migration on native PAGE is dependent on both mass and charge.

One possible solution to this problem would be to purify the Anbu complexes and analyze them by size exclusion chromatography. Using a size exclusion column, the size of the protein structures could be more accurately determined. Additionally, this analysis is much gentler on protein complexes than Native PAGE, which can result in the

dissociation of protein complexes. The observation that *P. aeruginosa* and *K. oxytoca* Anbu are running at different sizes may be the result of these conditions. If the *P. aeruginosa* Anbu normally exists as a double ring with weak trans-affinity between the rings, it may disassociate into single rings under Native PAGE, which would account for the difference in position of the two Anbu proteins when analyzed by Native PAGE.

Another factor that may have an adverse effect on assembly is the addition of the C-terminal tag used for protein purification. It may be possible that these tags are interfering with the interactions between individual monomers and result in a non-native assembly configuration. To address this concern, future experiments will attempt to purify an un-tagged version of Anbu for analysis.

## 4.2 Addressing Doublet Concerns

One of the primary concerns of this investigation was over the formation of the doublet when the *P. aeruginosa* Anbu protein was recombinantly expressed in *E. coli*. The most likely explanation was that Anbu protein was being clipped by a protease, resulting in two differently sized species. If this loss occurred at the N-terminus, the catalytic threonine would be removed resulting in a likely loss of activity.

The first attempt to address this issue was the addition of EDTA and HALT protease inhibitor cocktail to the lysis step of protein purification. These efforts failed to eliminate the doublet. In vitro expression of the Anbu protein was successful in shifting the doublet to a primarily top-heavy band orientation. However, the small amount of protein produced and the inability to concentrate the protein made this method of expression undesirable. Subsequent Western blot analyses of the *E. coli* produced Anbu

strongly suggested that the cleavage was C-terminal [Figures 9 and 10], resulting in a loss of the his-tag and perhaps a few additional C-terminal residues.

To further verify that the N-terminus was intact and not truncated, the more intense lower band was excised from a stained gel and analyzed by N-terminal Edman degradation by a commercial provider. The results of the sequencing revealed the authentic N-terminus was present, beginning with the critical Thr1 (data not shown). In addition to verifying the presence of the N-terminal Thr1, this analysis also removed another potential concern, namely the removal of the initiator methionine. Had this methionine been retained, it too would have resulted in a loss of any potential activity.

## 4.3 Activity

Over the course of this investigation, numerous activity assays have been performed with the intent of finding a substrate for Anbu. Model fluorogenic proteasome substrates were used to detect peptidase activity. Succinylated casein was tried as a loosely folded protein substrate for Anbu. The three small proteins α-lactalbumin, β-casein, and lysozyme were also analyzed as potential substrates. Anbu was analyzed with a commercially available peptide library to identify peptides to serve as substrates. Finally, K48 and K63 forms of diubiquitin were used as substrates in case the activity of Anbu was directed towards isopeptide bonds. None of these analyses were able to demonstrate that Anbu has catalytic activity. However, there are still more factors to be considered when trying to assess the potential catalytic function of Anbu.

One factor that may be responsible for the lack of activity is that the experimental conditions are sub-optimal for Anbu activity. Variables such as pH, metal ions, and salt

concentrations may need to be varied from those employed in the study in order to observe activity. Any residual nickel ions from the Ni-NTA column used to purify the protein could also inhibit function, although Flag-tagged Anbu also failed to demonstrate peptidase activity using the fluorogenic peptide substrates (data not shown). It will also be necessary to purify untagged Anbu and assay it for function to rule out the possibility that using C-terminally tagged Anbu might interfere with proper assembly and/or activity.

Additionally, it is also possible that another factor, such as a binding partner, is required for Anbu to be active. In the 20S core particle, the base of the 19S regulatory particle acts as an ATPase to allow for substrate entry and subsequent activity. In the HslVU complex, HslV assembles and the ATPase ring of HslU is needed for optimal functionality. Both of these structures require more than one type of subunit for correct assembly and subsequent activity. If Anbu is a proteasome, or at the very least a novel compartmentalized protease, it may require an ATPase ring in order to function. Since the experiments performed so far have only involved the Anbu protein, the lack of catalytic activity may be due to the lack of an equivalent ATPase structure.

It is worthwhile to pursue potential binding partners as a means of Anbu activation. Preliminary pull-down assays have indicated that the native Anbu structure may be associating with other proteins in *P. aeruginosa* (Lindsay Hammack, unpublished). Moreover, in the bacteria that have it, Anbu is associated with an operon of three conserved but uncharacterized proteins which have been hypothesized to function with Anbu (*39*). It would be worthwhile to investigate these proteins to determine if they have a role in the function of Anbu. Expression and purification of these proteins may

yield further insight into the function of Anbu and the currently uncategorized proteins. Should a definite binding partner be identified, experiments such as the activity assays will need to be repeated.

## 4.4 Mutant Analysis

The comparative analysis between the insertion mutants and WT PA14 has yielded interesting results. All of the strains containing the insertional mutants were still viable, which indicates that none of these proteins are essential to the organism. Western analysis of the cell lysate has indicated that the Anbu protein is expressed and can be detected when cultures are grown at 37 °C in LB. This indicates that the function of Anbu may be related to general homeostasis, as opposed to being regulated and expressed only under specific conditions. It is also worth mentioning that the mutant HslV strain does not have an obvious increase in Anbu expression. If the functions were partially redundant, then the loss of one may cause an over-expression in the other as a means of compensation. This indicates that the two proteins may have two independent roles in the organism.

When the other mutants were examined by Western analysis, the respective proteins of interest could not be identified in the cell lysate. There are a number of potential explanations as to why these proteins could not be detected. It is possible that the antibodies are able to recognize the recombinant proteins and not recognize their native counterparts due to post-translational modification of the *Pseudomonas* proteins. Alternatively, these proteins may have short half-lives and are rapidly degraded shortly after translation. The levels of expression could be low enough such that the signal could

not be developed properly. Another explanation for the lack of detection is that these proteins are not expressed during the normal growth conditions of 37 °C in LB. It would be worthwhile to perform Western analysis on stressed cells in further attempts to detect these proteins.

The sensitivity of the mutant Anbu strain of *P. aeruginosa* to agents such as diamide and s-nitrosoglutathione (GSNO) are intriguing, but not entirely surprising. These agents can impart oxidative (and/or nitrosative in the case of GSNO) stress upon cells, one consequence of which could be the misfolding of oxidatively damaged proteins. In the absence of Anbu, growth of *P. aeruginosa* is negatively affected. These results indicate that Anbu has some role in alleviating oxidative/nitrosative stress. In addition to the Anbu mutant being sensitive to the conditions tested, the HslV mutant was similarly affected. These results indicate that both proteins have a role in relieving the stress caused by diamine and s-nitrosoglutathione. Further experiments will need to be performed to investigate this phenomenon further.

Of immediate priority is a complementation assay using a *P. aeruginosa* expression plasmid bearing Anbu (*60*). This would verify that the impaired growth seen in the Anbu mutant is indeed caused by lack of Anbu. Other stresses such as high salt concentration, heavy metals and temperature could likewise be tested.

An indirect assay for proteolysis could be to carry out a complementation assay using a plasmid-borne T1A mutant of Anbu. If the N-terminal threonine is catalytic, such a mutation should fail to rescue the aforementioned growth phenotypes. Another interesting experimental design would be a double knockout of the HslV and Anbu

proteins. With no proteasome-like structures, this mutant would likely be highly sensitive to stress conditions, if it is even viable.

Another interesting point of investigation would be to determine cell viability at the end of the stress condition assays. In a number of the stress conditions, culture density leveled off prematurely. If aliquots from these cultures were plated after analysis it could be determined if this lack of growth is due to cell death.

## 4.5 Closing Thoughts

Much experimental work has been performed in order to determine if Anbu is a rightful member of the proteasome family. While experimental results show that Anbu is assembling into a large structure and has a role in handling stress from agents that impart oxidative/nitrosative stress, a substrate has yet to be found. As of this writing, it cannot be definitively stated whether Anbu is or is not a proteasome. However, there are still a multitude of experiments that can be performed to further explore questions surrounding this potential proteasome homologue. Even if Anbu is shown to not function as a proteasome, its clear sequence homology to proteasome subunits, assembly, and role in handling stress will help shed much needed light on the evolution of proteasome genes.

TABLES

Table 1: **Primers Used in Cloning Experiments:**

| Primer Function | Sequence 5′-3′ |
|---|---|
| Anbu Forward | GCATATGACCTACTGTGTCGCGATG |
| Anbu Reverse | TCTCGAGTCAGATGTTGTACGCCGAGGGCGGCG |
| Anbu Reverse + His-tag | TCTCGAGTCAATGGTGATGGTGATGGTGGGATCCGATGTTGTACGCCGAGGGCGGCG |

FIGURES

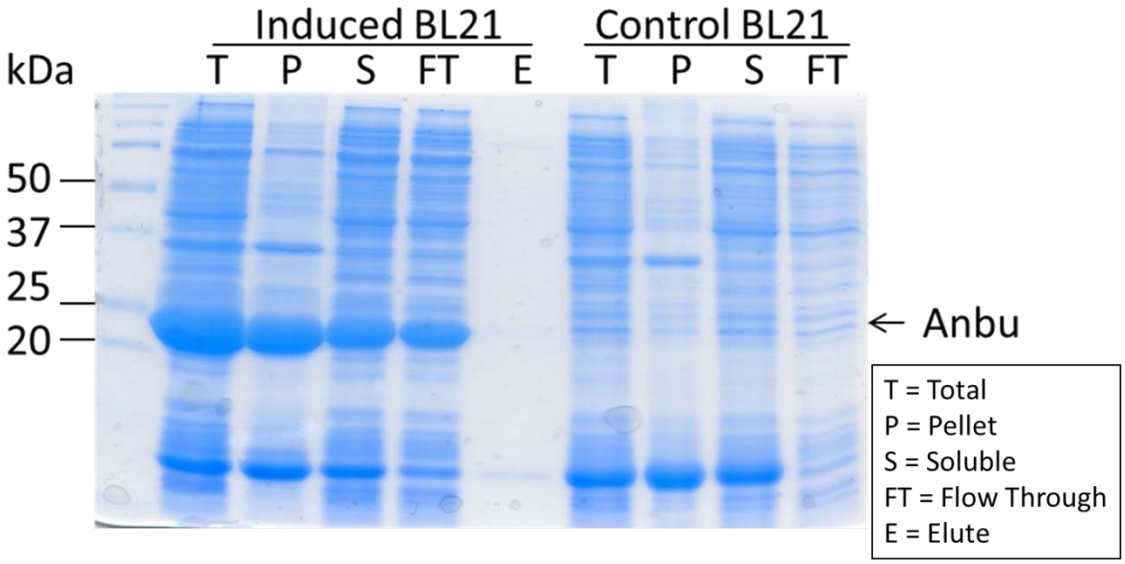



Figure 1: **Fractional Analysis of BL21 Cells Expressing Anbu from *P. aeruginosa*:** 12% SDS-PAGE analysis of fractionation and protein purification of the non-tagged Anbu protein cloned from *P. aeruginosa*. Fractions were also run from BL21 cells not expressing protein (Control BL21).

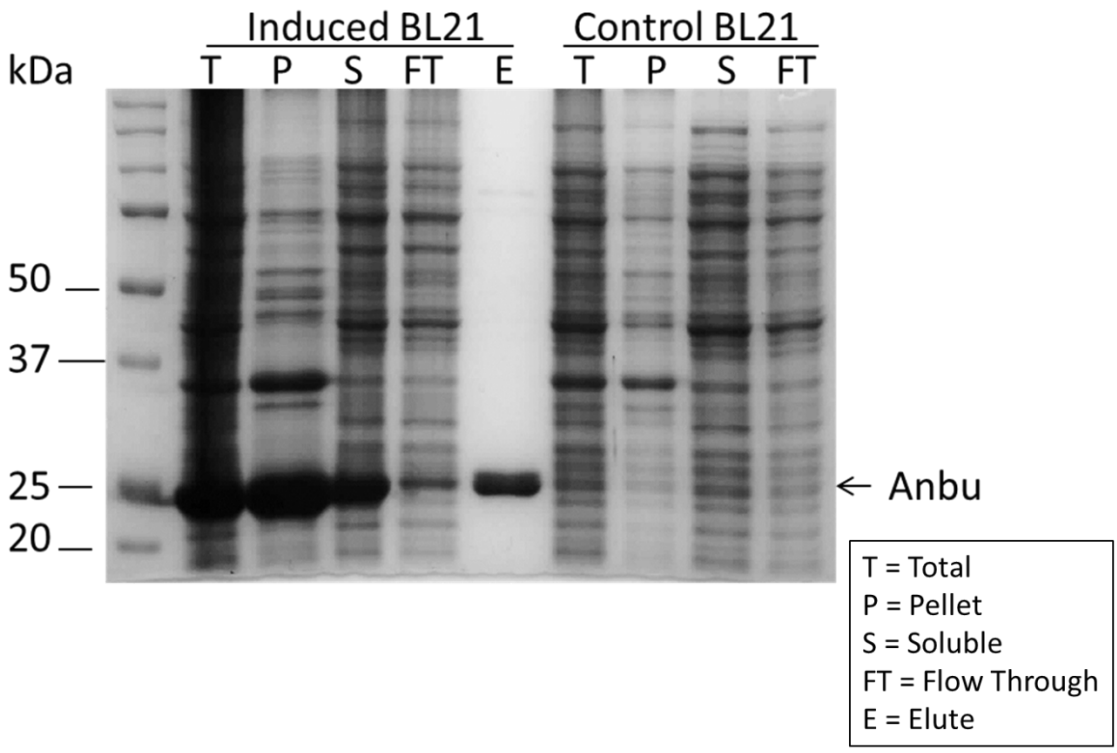



Figure 2: **Fractional Analysis of BL21 Cells Expressing Hexahistidine Tagged Anbu from *P. aeruginosa*:** 10% SDS-PAGE analysis of fractionation and protein purification of the hexahistidine tagged Anbu protein cloned from *P. aeruginosa*. Fractions were also run from BL21 cells not expressing protein (Control BL21).

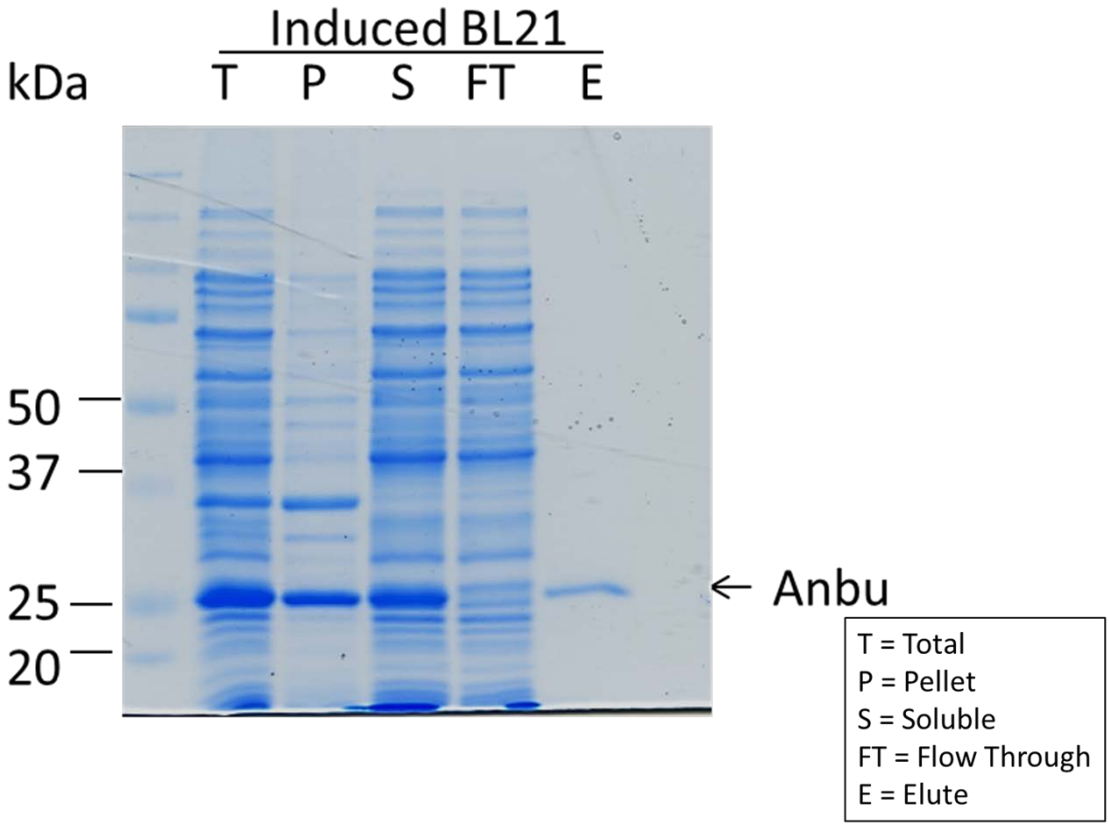



Figure 3: **Fractional Analysis of BL21 Cells Expressing Hexahistidine Tagged Anbu from *K. oxytoca*:** 10% SDS-PAGE analysis of fractionation and protein purification of the hexahistidine tagged Anbu protein cloned from *K. oxytoca*.

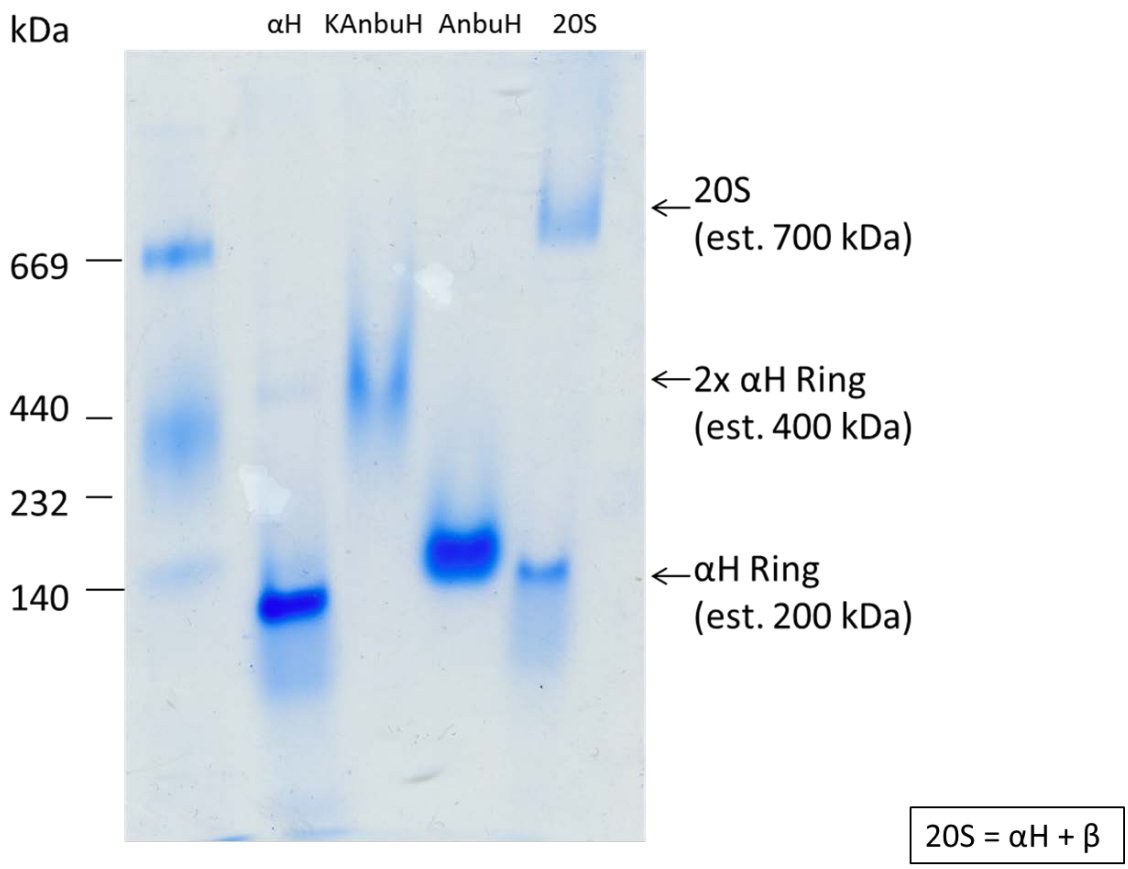



Figure 4: **Native PAGE Analysis of the Recombinant Hexahistidine Tagged Anbus from *K. oxytoca* and *P. aeruginosa*:** Native PAGE analysis for hexahistidine tagged Archaeal α-subunits (αH), hexahistidine tagged Anbu from *K. oxytoca* (KAnbuH), hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), Archaeal proteasomes (20S).

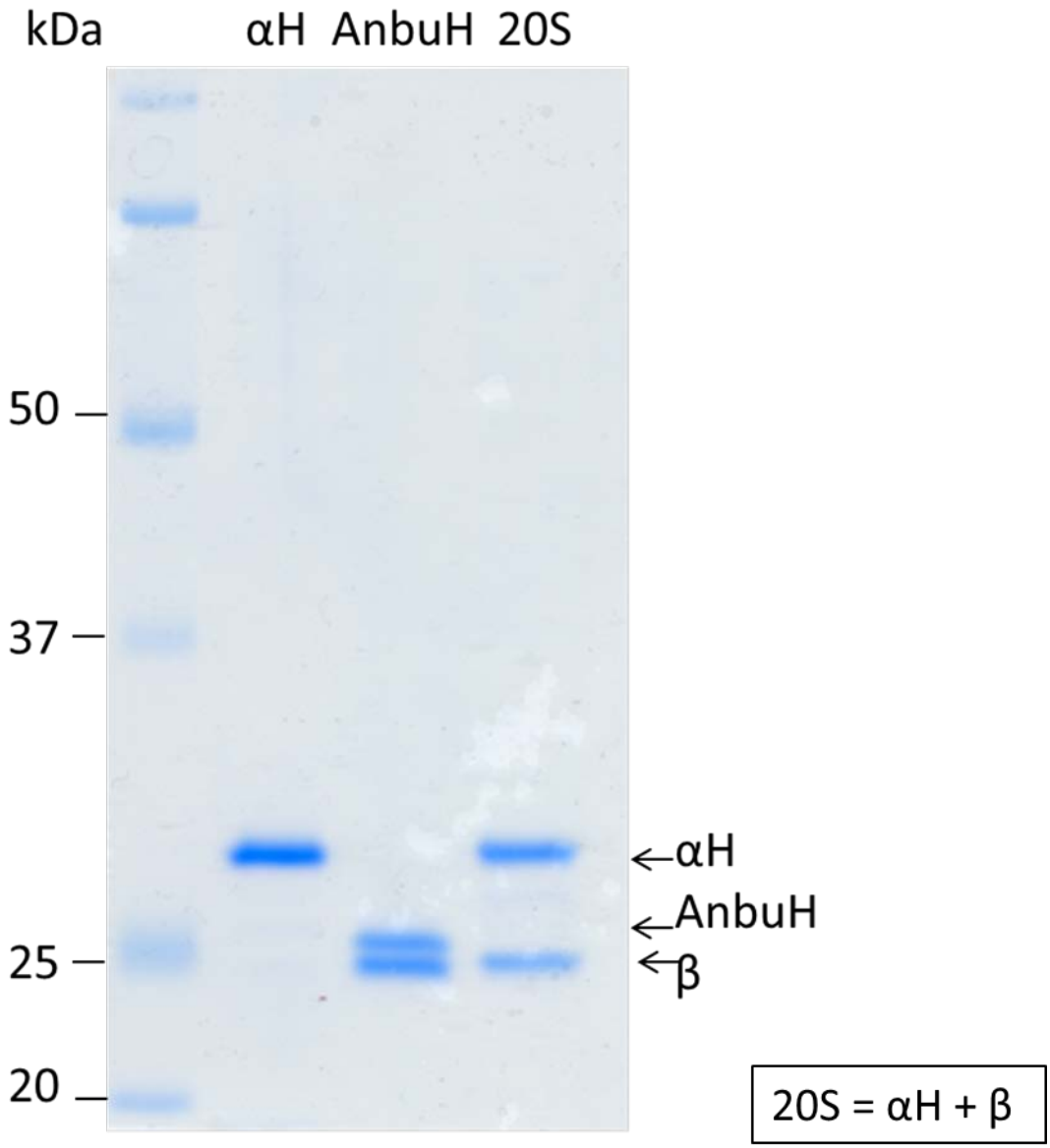



Figure 5: **Large SDS-PAGE Analysis of the Hexahistidine Tagged Anbu from *P. aeruginosa* as an Attempt to Resolve the Doublet:** Large SDS-PAGE containing Archaeal α-subunits (αH), hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and Archaeal proteasome (20S).

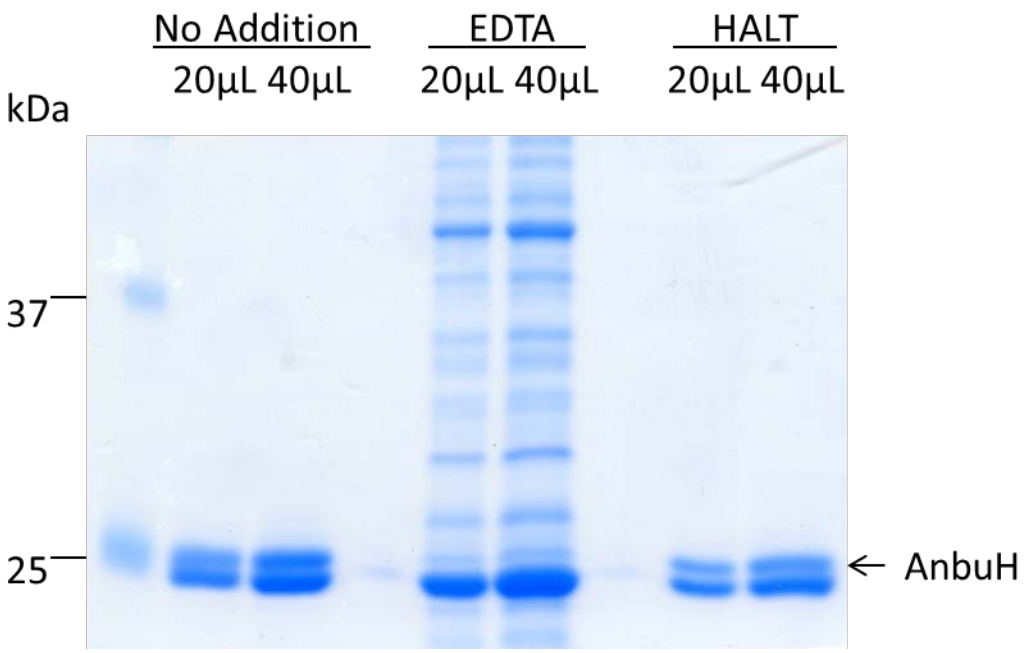



Figure 6: **Large SDS-PAGE of Hexahistidine Tagged Anbu from *P. aeruginosa* with EDTA and HALT Additions:** Large SDS-PAGE with samples of Anbu purified under standard lysis protocol and with the addition of HALT Protease Inhibitor Cocktail. EDTA lanes contain the soluble lysate of induced cells.

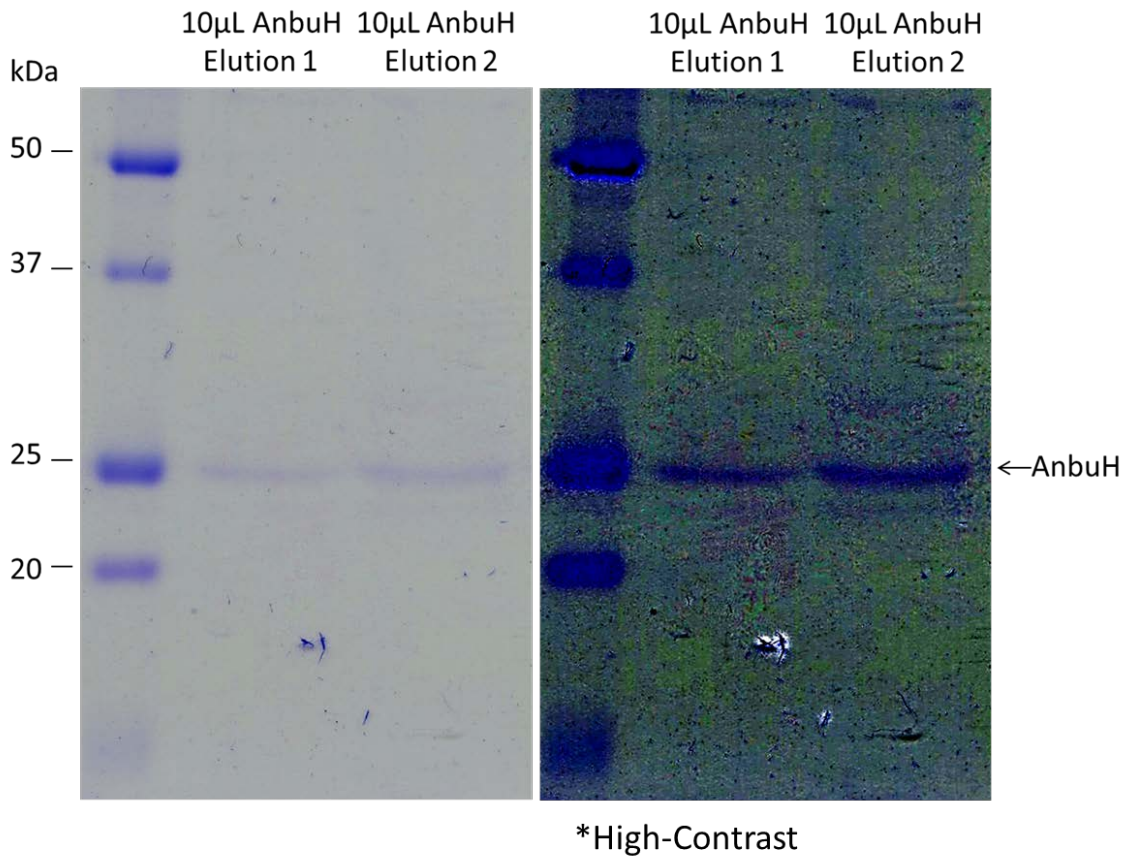



Figure 7: **SDS-PAGE of the Hexahistidine Tagged Anbu from *P. aeruginosa* Produced with the In-Vitro Expression Kit:** 12% SDS-PAGE of elution fractions from the EasyXpress kits that produced the hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH) (left). The contrast has been altered to show the faint lower band of the doublet (right).

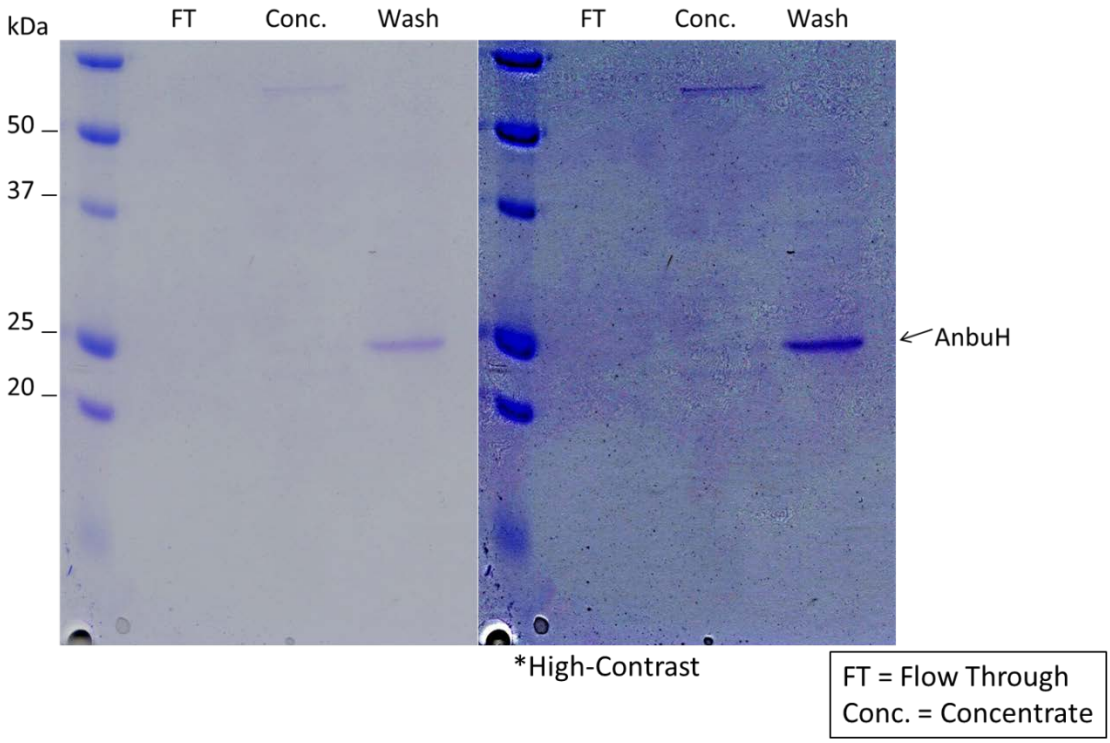



Figure 8: **SDS-PAGE of the Concentration Fractions of the Hexahistidine Tagged Anbu from *P. aeruginosa* Produced with the In-Vitro Expression Kit:** 12% SDS-PAGE analysis of concentrator fractions from the Flow Though (FT), concentration (Conc.), and Wash of the concentrator membrane. These aliquots were taken as they were produced in the concentrators after the hexahistidine tagged Anbu from *P. aeruginosa* had been produced in the EasyXpress kits and purified. Regular scan is on the left, high-contrast image showing the light doublet in the concentrate lane on the right.

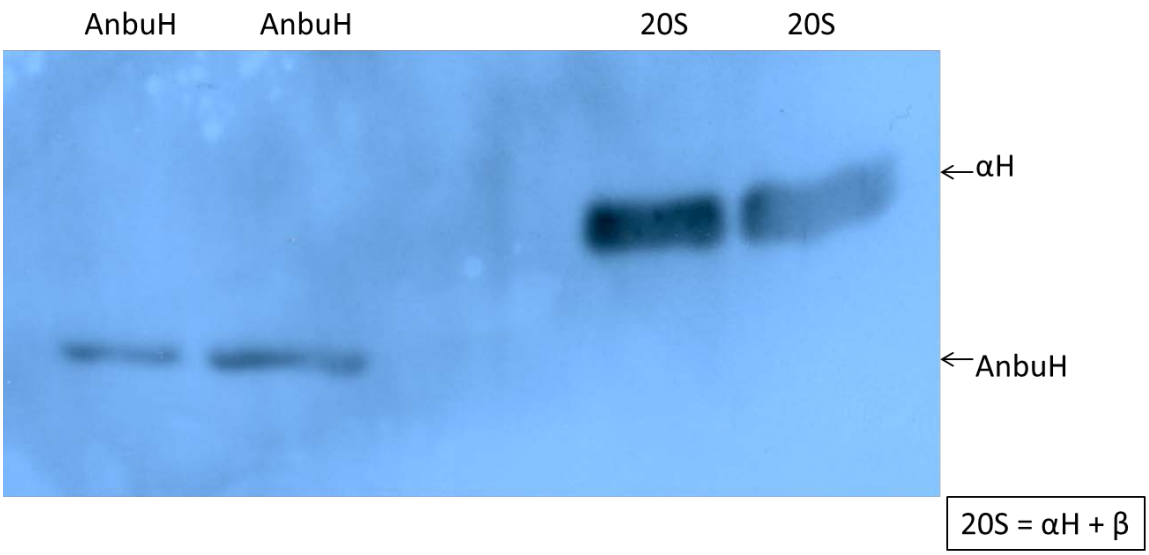



Figure 9: **Anti Hexahistidine Tag Western of Hexahistidine Tagged Anbu from *P. aeruginosa*:** Anti his-tagged Western blot from a large SDS-PAGE. Signal from the AnbuH lanes is a singlet. Positive control is the Archaeal proteasome (20S) containg a hexahistidine tagged α-subunit (αH).

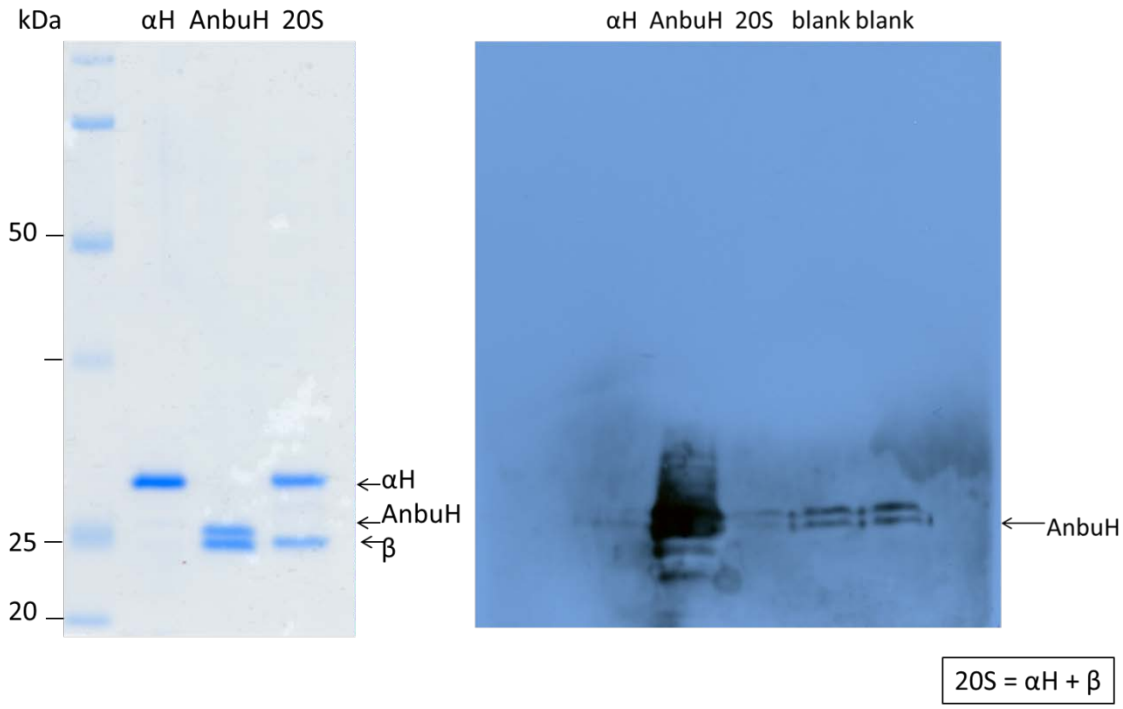



Figure 10: **Anti Anbu Western of Hexahistidine Tagged Anbu from *P. aeruginosa*:** Large SDS-PAGE for the negative control Archaeal α-subunits (αH), experimental sample was hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and the positive control Archaeal proteasome (20S) (left). Anti-Anbu Western blot for samples of Archaeal α-subunits (αH), hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and Archaeal proteasomes (20S) (right). Signal is strong in AnbuH lane. AnbuH overflow contaminated lanes that were loading buffer only, and the bands are resolved as a doublet (right).

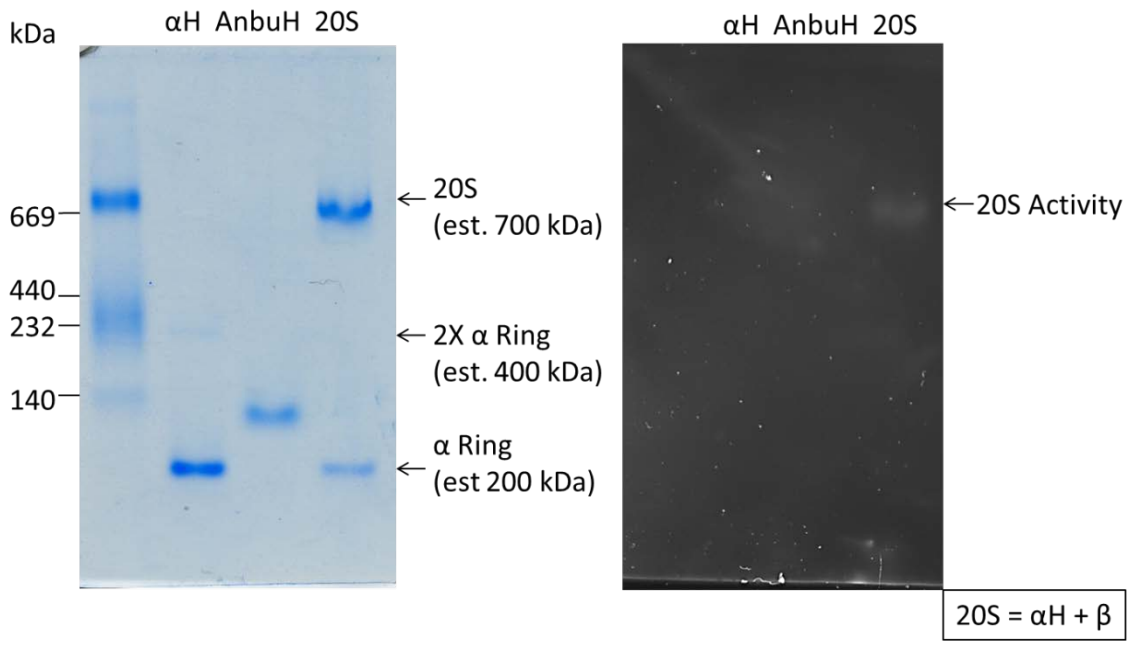



Figure 11: **In-Gel Activity Assay of Hexahistidine Tagged Anbu from *P. aeruginosa*:** Activity assay with the Suc-LLVY-AMC substrate on a Native PAGE. The negative control was Archaeal α-subunits (αH), experimental sample was hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and the positive control Archaeal proteasome (20S). Fluorescence from activity was visualized with UV (right) and then stained with GelCode Blue (left).

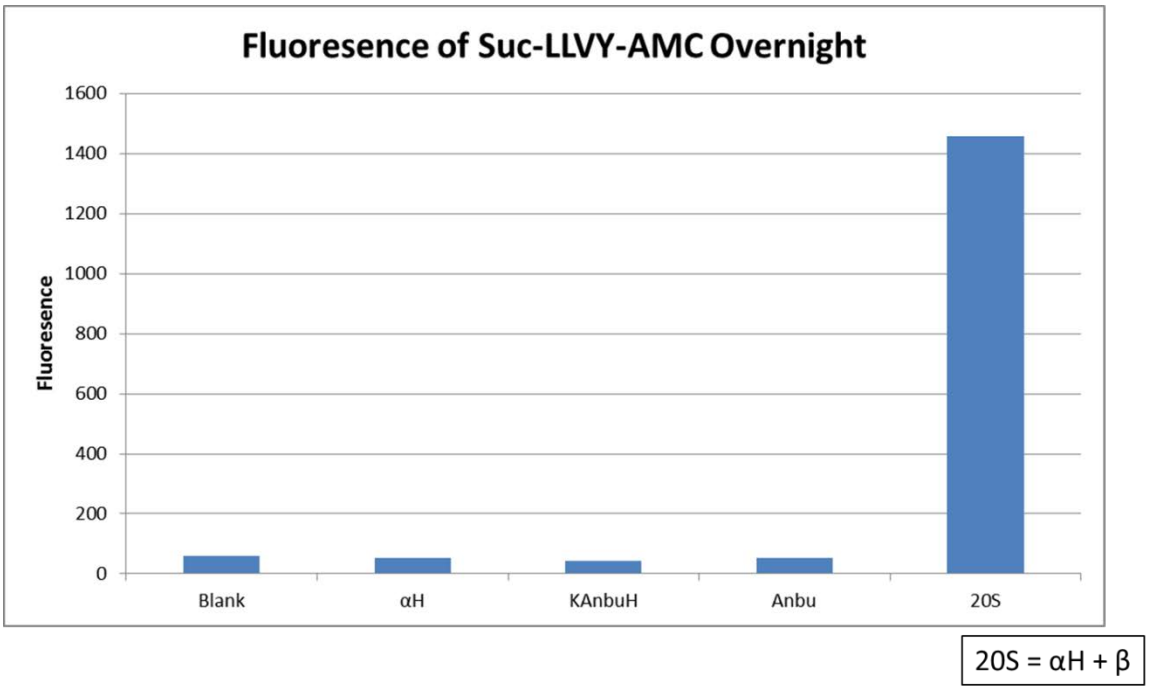



Figure 12: **Overnight Fluorescent Activity Analysis of Suc-LLVY-AMC with the Recombinant Anbus:** Spectrophotometer readings of Suc-LLVY-AMC activity analysis performed in a microtiter plate overnight. The negative control was Archaeal α-subunits (αH), experimental samples were hexahistidine tagged Anbu from *K. oxytoca* (KAnbuH) and hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and the positive control was the Archaeal proteasome (20S). Fluorescence readings in relative fluorescence units (RFUs) greater than the blank indicate activity.

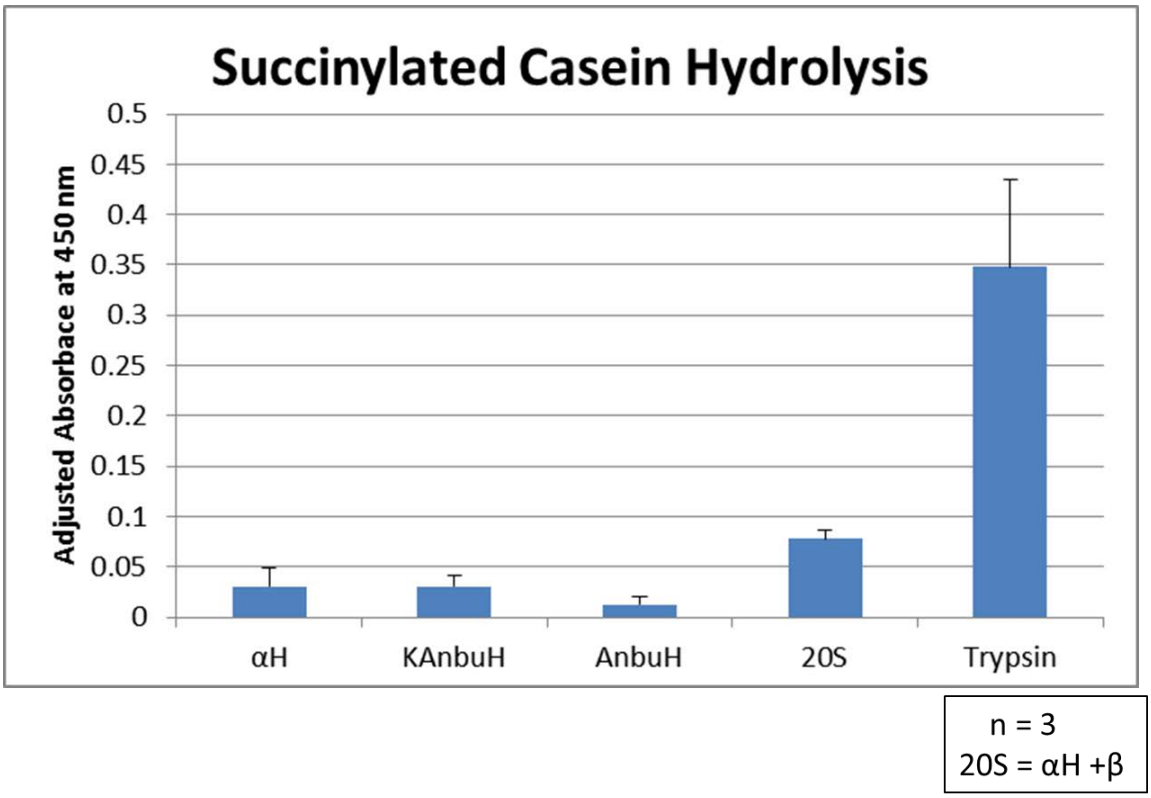



Figure 13: **Protease Activity of Succinylated Casein with the Recombinant Anbus:** Spectrophotometer readings indicating color change in the QuantiCleave assay. Higher absorbance indicates higher activity. Adjusted absorbance was calculated by subtracting the absorbance readings obtained from a blank lacking one of the variable proteins from the experimental and control readings. The negative control was Archaeal α-subunits (αH). Experimental samples were hexahistidine tagged Anbu from *K. oxytoca* (KAnbuH) and hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH), and the Archaeal proteasome (20S). The positive control for this experiment was Trypsin.

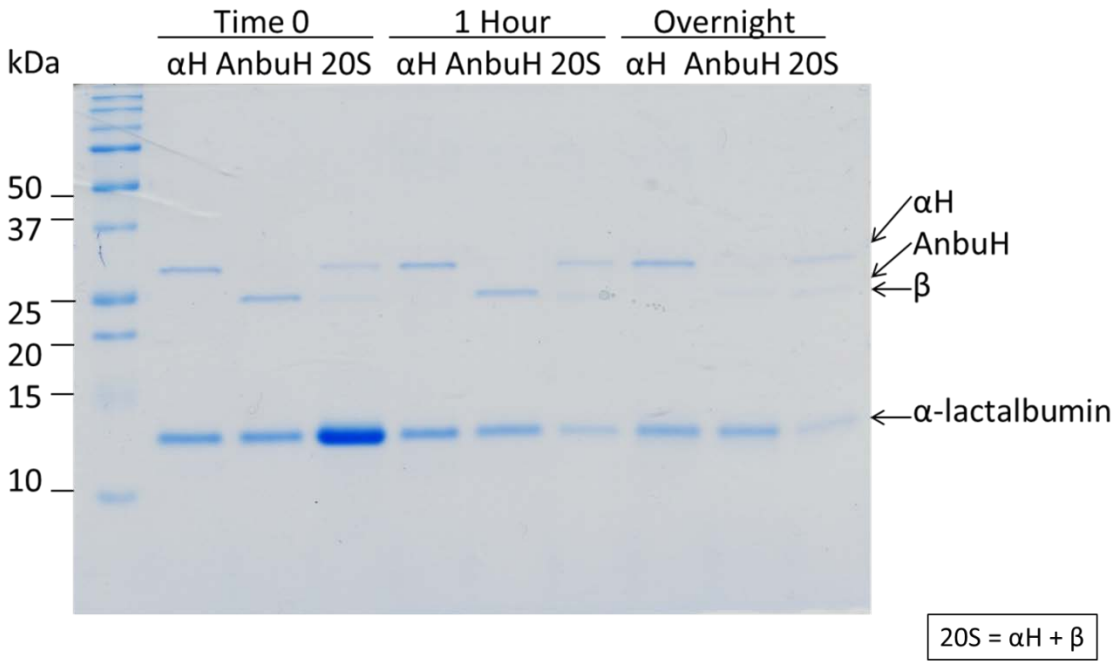



Figure 14: **SDS-PAGE of Small Protein Degradation Assay Indicating Protease Activity with α-Lactalbumin:** Samples of the potential substrate of denatured α-lactalbumin were incubated overnight with the negative control Archaeal α-subunits (αH). Experimental samples were hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH) and Archaeal proteasomes (20S) incubated with the denatured protein. Aliquots were taken at time points 0, 1 hour and overnight. Samples were then analyzed by SDS-PAGE to detect degradation of the small proteins.

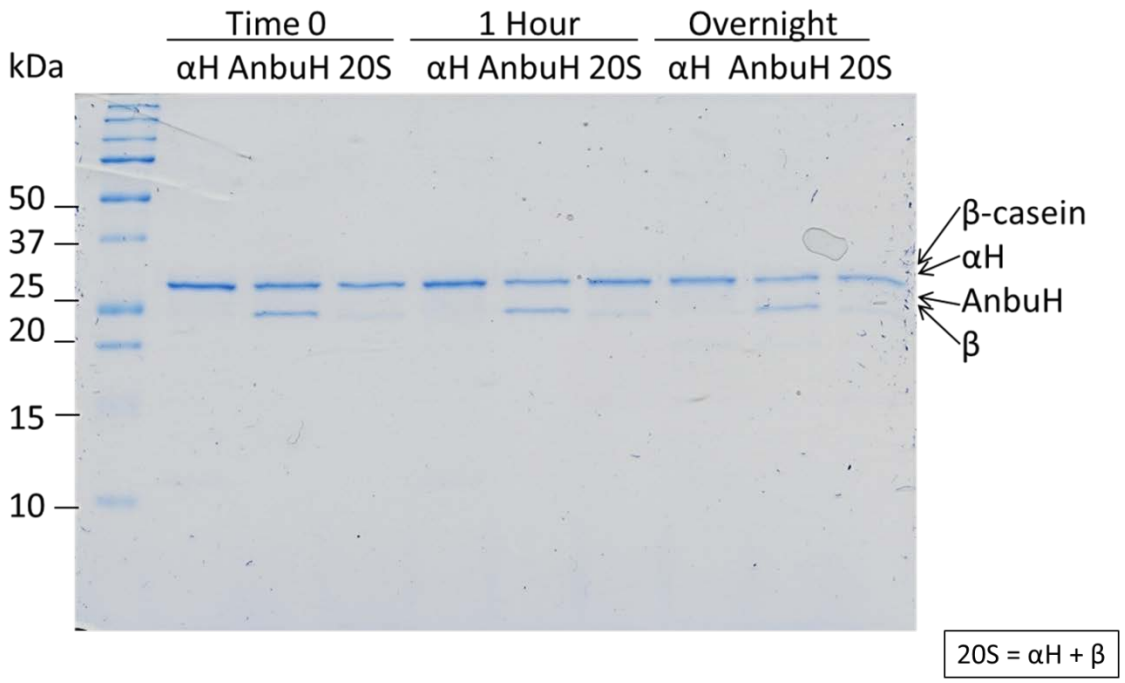



Figure 15: **SDS-PAGE of Small Protein Degradation Assay Indicating Protease Activity with of β-Casein:** Samples of the potential substrate of denatured β-casein were incubated overnight with the negative control Archaeal α-subunits (αH). Experimental samples were hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH) and Archaeal proteasomes (20S) incubated with the denatured protein. Aliquots were taken at time points 0, 1 hour and overnight. Samples were then analyzed by SDS-PAGE to detect degradation of the small proteins. NOTE: Archaeal α-subunits (αH) and β-Casein are close in size and running at an almost identical position.

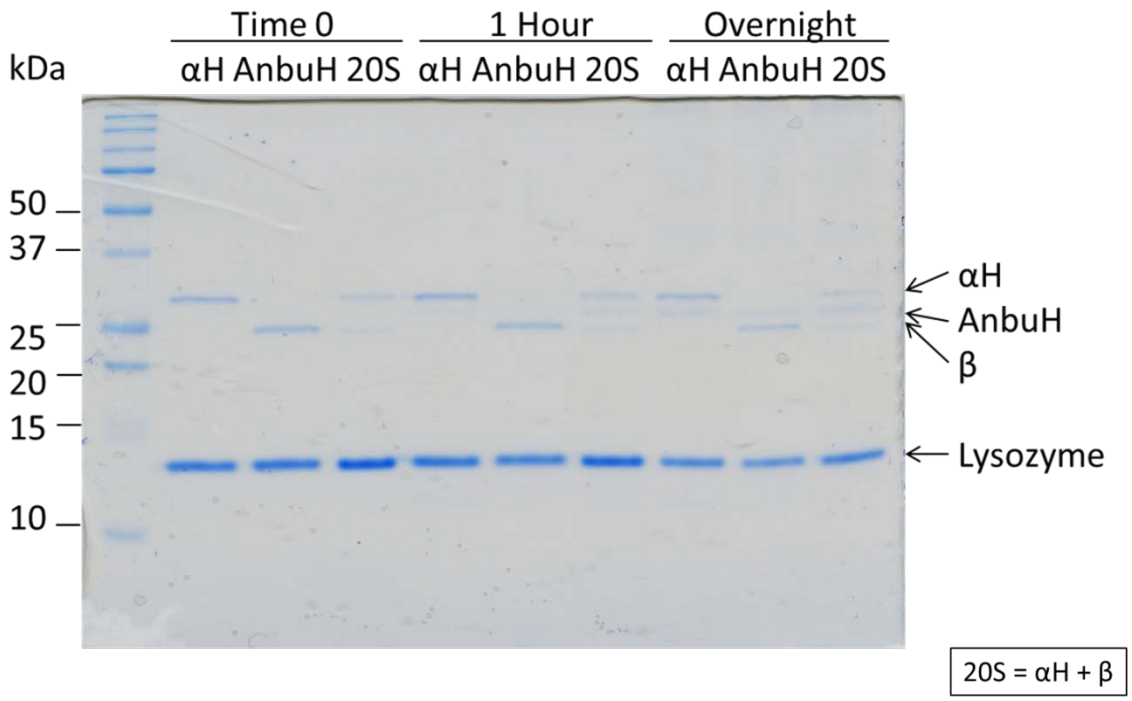



Figure 16: **SDS-PAGE of Small Protein Degradation Assay Indicating Protease Activity with of Lysozyme:** Samples of the potential substrate of denatured lysozyme were incubated overnight with the negative control Archaeal α-subunits (αH). Experimental samples were hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH) and Archaeal proteasomes (20S) incubated with the denatured protein. Aliquots were taken at time points 0, 1 hour and overnight. Samples were then analyzed by SDS-PAGE to detect degradation of the small proteins.

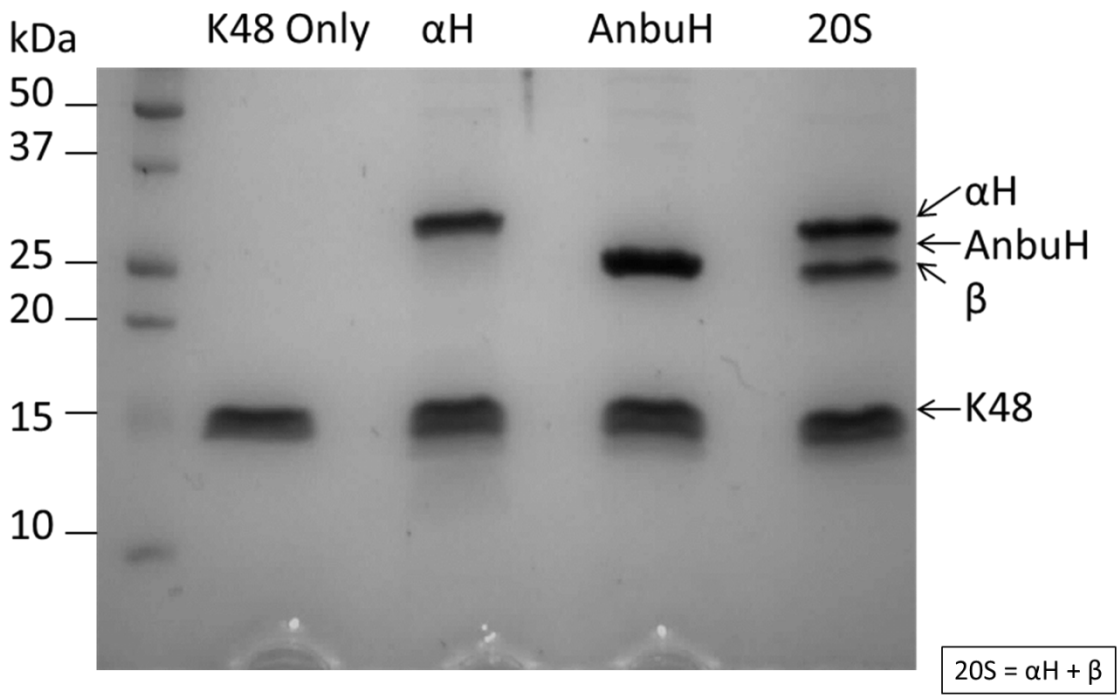



Figure 17: **Isopeptidase Activity with K48 Linked Diubiquitin:** 15% SDS-PAGE of the overnight incubations of K48 linked diubiquitin. The loading control contained K48 diubiquitin only. The negative controls were Archaeal α-subunits (αH) and the Archaeal proteasome (20S). The experimental sample was hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH).

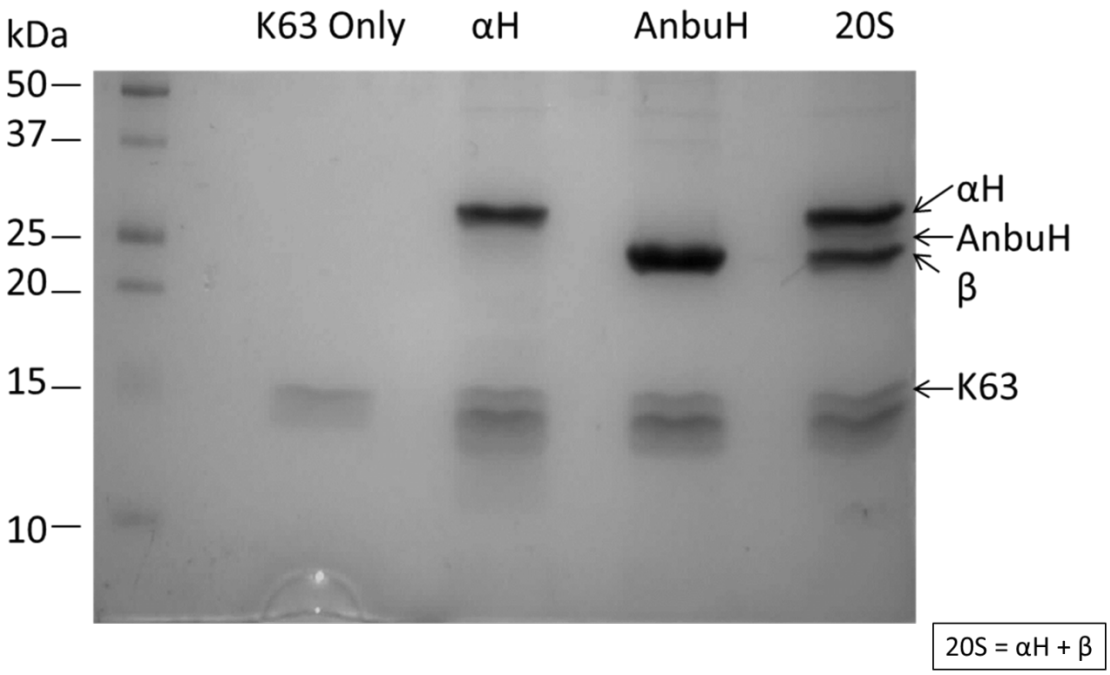



Figure 18: **Isopeptidase Activity with K63 Linked Diubiquitin:** 15% SDS-PAGE of the overnight incubations of K63 linked diubiquitin. The loading control contained K63 diubiquitin only. The negative controls were Archaeal α-subunits (αH) and the Archaeal proteasome (20S). The experimental sample was hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH).

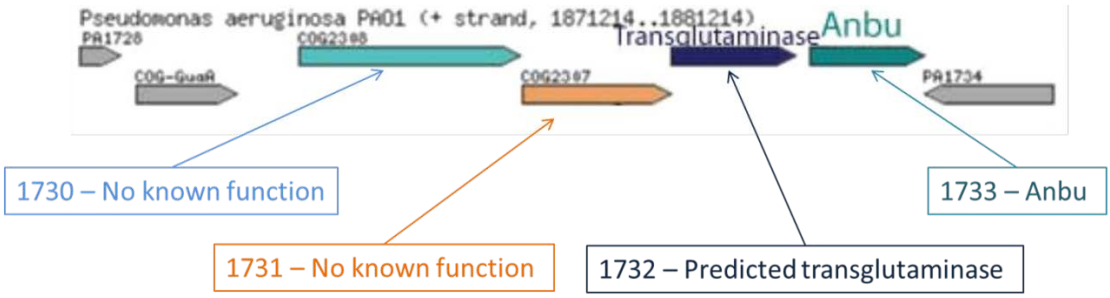



Figure 19: **Anbu Operon Configuration from *P. aeruginosa* Strain PA01:** Annotated figure from the Valas 2008 publication showing the orientation of the predicted Anbu operon in *P. aeruginosa* strain PA01. Coding regions for genes 1730 and 1731 have no predicted function. Gene 1732 is a predicted transglutaminase. Gene 1733 is the Anbu protein. Transposon insertional mutants into each of these genes serve as the mutants of interest in following experiments.

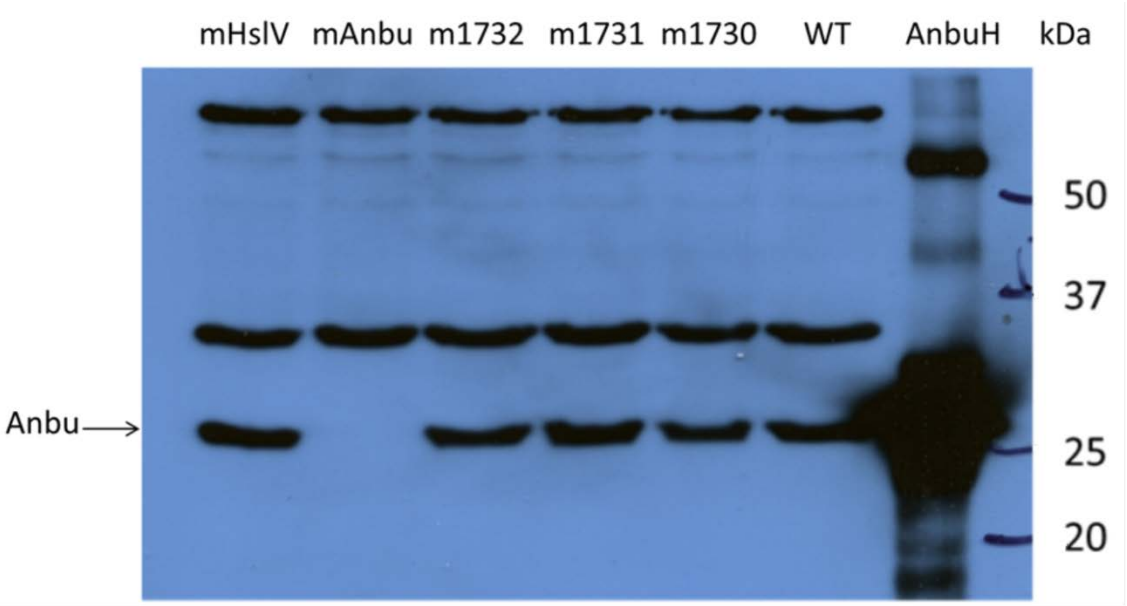
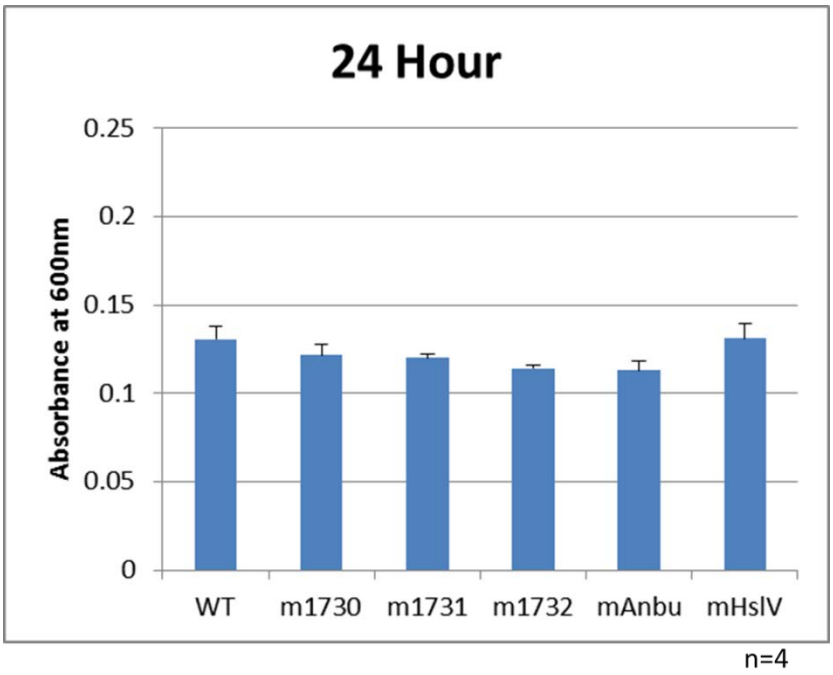



Figure 20: **PA14 and Insertional Mutants of Interest Whole Cell Lysate Probed for Anbu by Western Blot:** Western blot of WT PA14 and mutants of interest probed with the polyclonal anti-Anbu Antibody. Recombinant hexahistidine tagged Anbu from *P. aeruginosa* (AnbuH) served as the positive control.



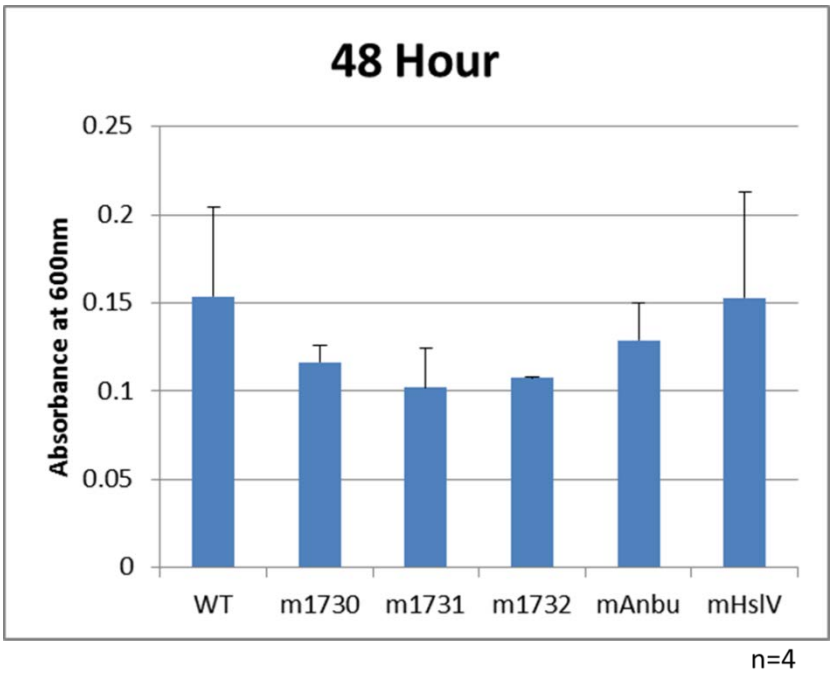




Figure 21: **Biofilm Formation in PA14 and Insertional Mutants of Interest:** Absorbance readings from the biofilm formation assay. Higher absorbance readings from the spectrophotometer indicate higher biofilm production. Data from 24 hour analysis shown above and 48 hour data is below.

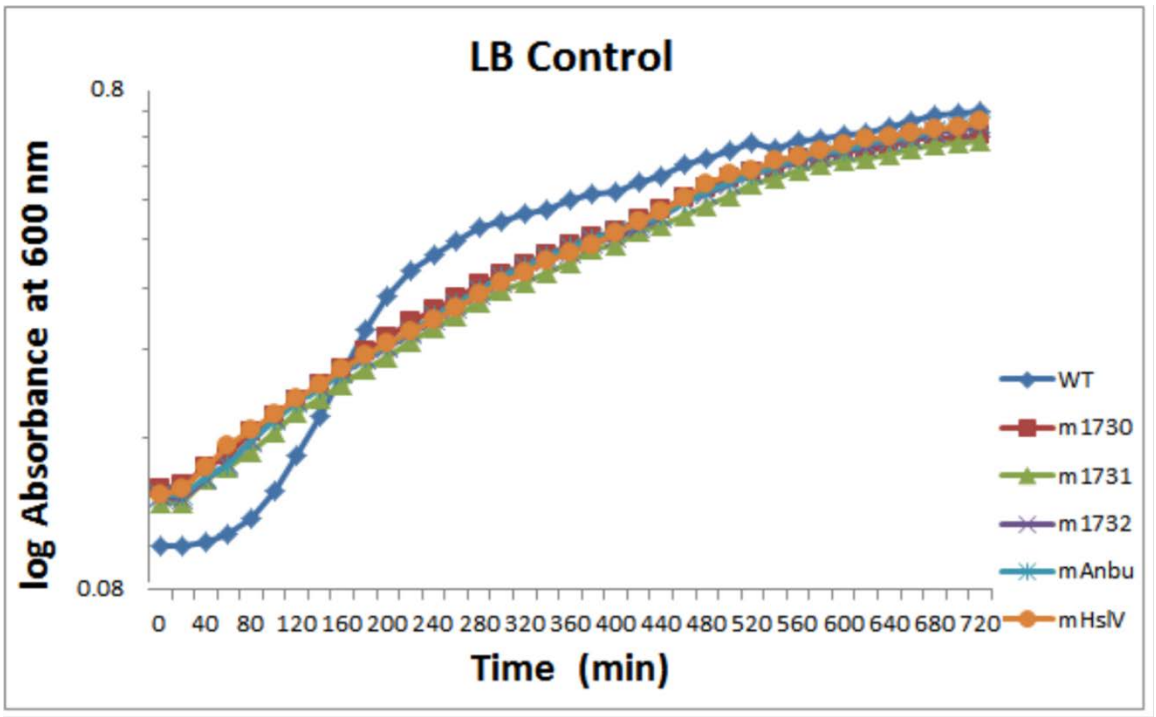



Figure 22: **LB Growth Curve Control for PA14 and Insertional Mutants of Interest from Diamide Analysis:** Growth curve control of all cultures analyzed grown in LB with no additives. Cultures were analyzed every 20 minutes for a total of 12 hours at 37 °C with agitations before readings. Samples were analyzed in duplicate and growth curves are representative of 3 separate experiments.

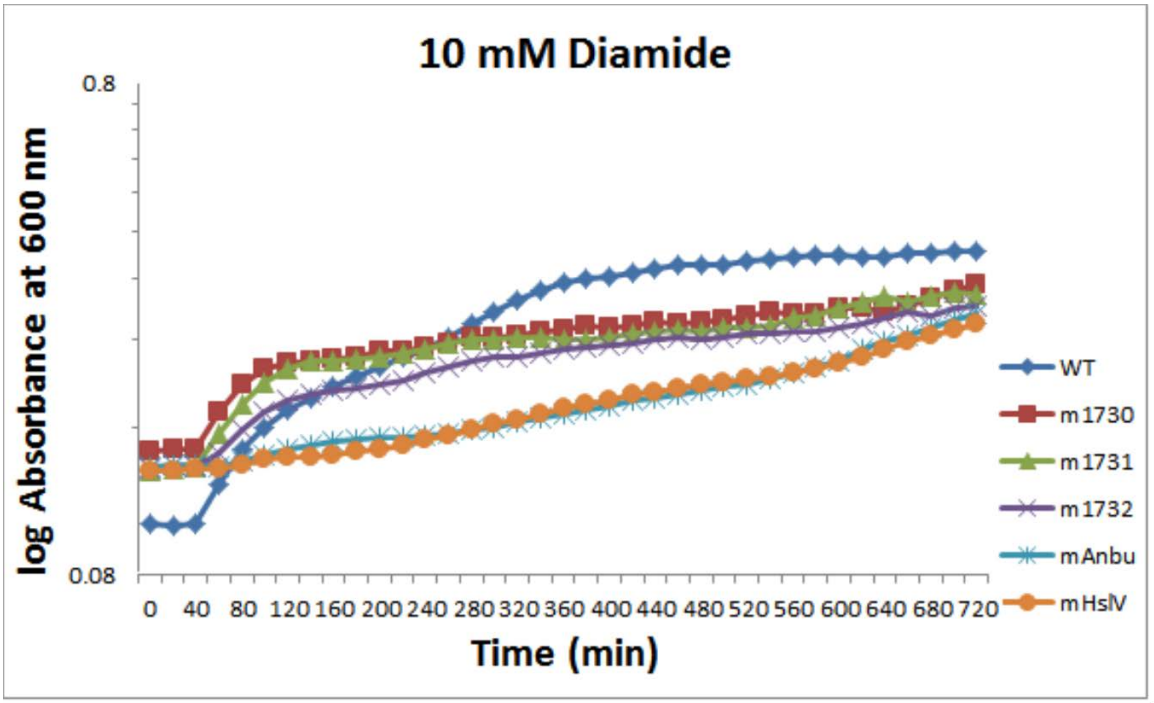



Figure 23: **10 mM Diamide Growth Curve for PA14 and Insertional Mutants of Interest:** Growth curve control of all cultures analyzed grown in LB with a final concentration of 10 mM Diamide. Cultures were analyzed every 20 minutes for a total of 12 hours at 37 °C with agitations before readings. Samples were analyzed in duplicate and growth curves are representative of 2 separate experiments.

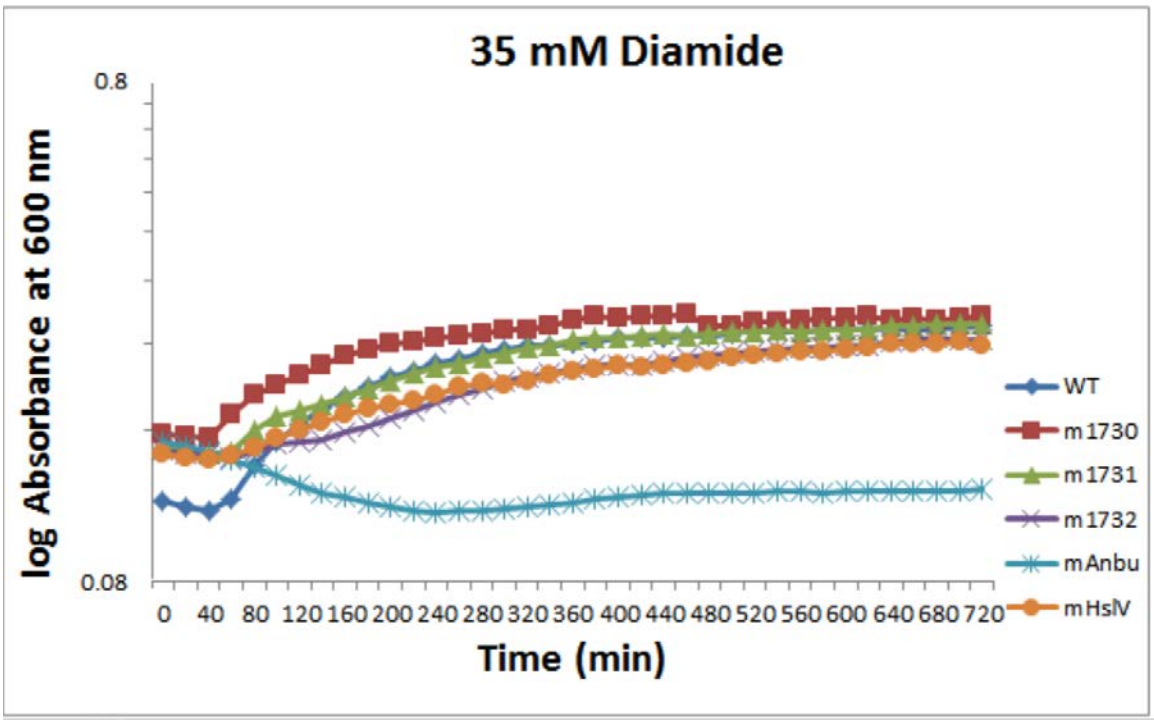



Figure 24: **35 mM Diamide Growth Curve for PA14 and Insertional Mutants of Interest:** Growth curve control of all cultures analyzed grown in LB with a final concentration of 35 mM diamide. Cultures were analyzed every 20 minutes for a total of 12 hours at 37 °C with agitations before readings. Samples were analyzed in duplicate and growth curves are representative of 2 separate experiments.

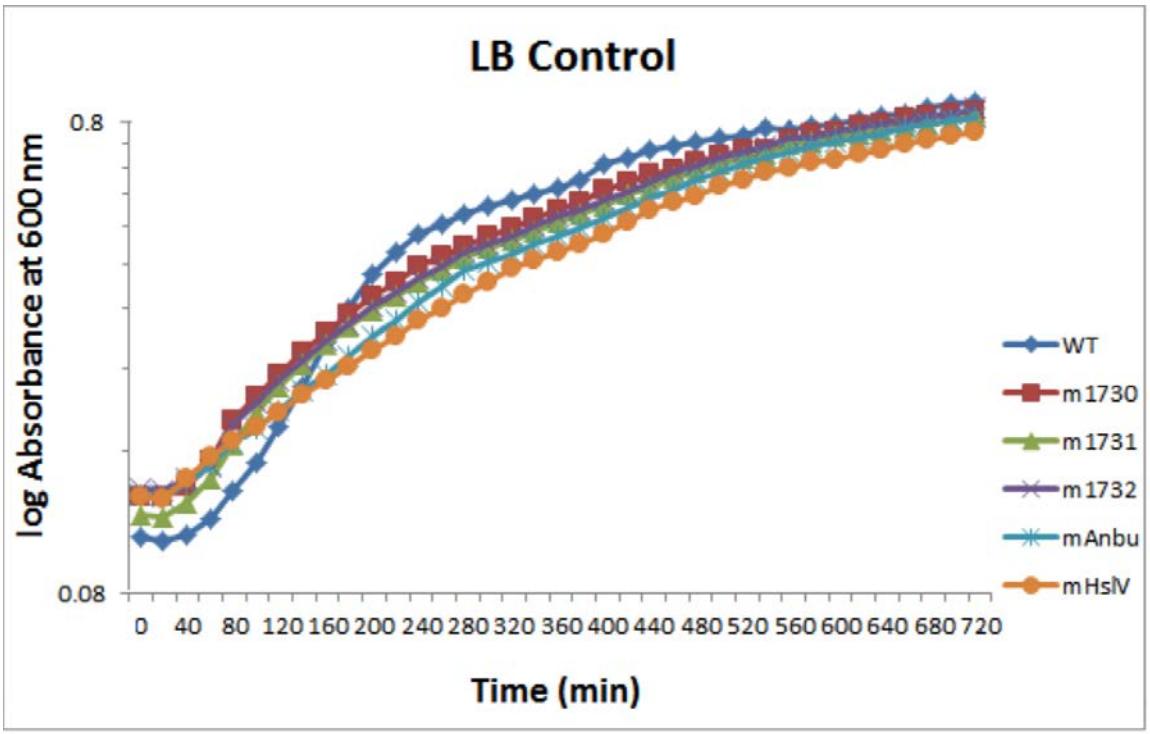



Figure 25: **LB Growth Curve Control for PA14 and Insertional Mutants of Interest from S-nitrosoglutathione Analysis:** Growth curve control of all cultures analyzed grown in LB with no additives. Cultures were analyzed every 20 minutes for a total of 12 hours at 37 °C with agitations before readings. Samples were analyzed in duplicate and growth curves are representative of 3 separate experiments.

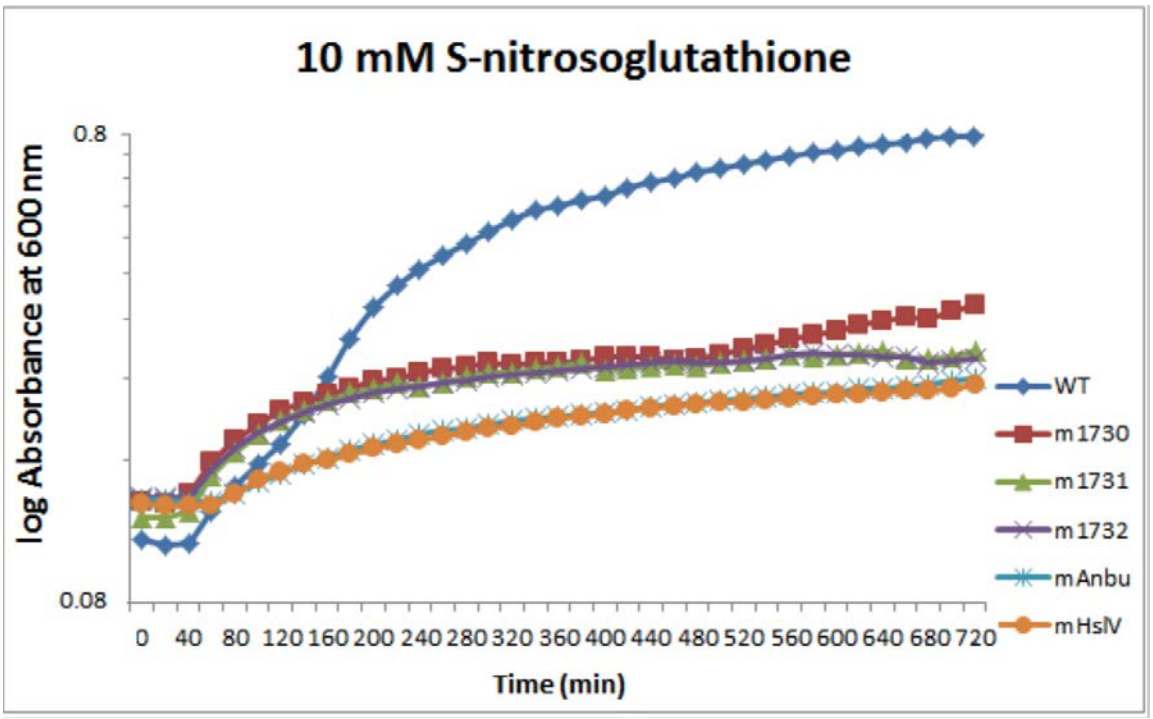



Figure 26: **10 mM S-nitrosoglutathione Growth Curve for PA14 and Insertional Mutants of Interest:** Growth curve control of all cultures analyzed grown in LB with S-nitrosoglutathione added to a final concentration of 10 mM. Cultures were analyzed every 20 minutes for a total of 12 hours at 37 °C with agitations before readings. Samples were analyzed in duplicate and growth curves are representative of 3 separate experiments.

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# WORKS CITED


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