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Protease From a Novel Antarctic Bacterial Isolate

by

Brittany A. Mangum

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in the Medical Laboratory Science

Idaho State University

August 2023

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ACKNOWLEDGEMENTS

This project would not have been feasible without the support of many people. Thanks are due to Dr. Peter Sheridan, who provided guidance and support throughout my research project and helped revising this document. To my family and friends, thank you for your support and encouragement.

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List of Abbreviations

PDA	Potato Dextrose Agar
LB	Luria-Bertani
MM	Minimal milk
K_{cat}	Specific activity
K_m	Michaelis-Menten constant
K_{cat}/K_m	Catalytic efficiency
PB	Phosphate buffer
FBS	Fetal bovine serum
AFP	Antifreeze proteins
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulfate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
DTT	Deep vein thrombosis
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
LB	Luria-Bertani
LIC	Ligation Independent Cloning
P5	Protease 5
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride

RSS	Rapid Silver Stain
SP1	Serine Protease 1
X-gal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside
ZnP2	Zinc Protease 2

Protease From a Novel Antarctic Bacterial Isolate

Thesis Abstract-Idaho State University (2022)

Son of Salt (SOS) Pond novel isolate, *Peribacillus frigotolerans,* from a hypersaline pond located in the McMurdo Ice Shelf in Antarctica has shown to produce proteases and β-galactosidases. We have identified three proteases (serine protease 1, protease 5, and zinc protease 2) and designed PCR primers to amplify the genes. Utilizing the aLICator cloning system, we were able to clone the three gene targets. Additionally, we have transformed recombinant plasmid DNA into an expression host and preliminary expression experiments have been conducted.

Keywords: *Peribacillus*, cold-adapted, protease, β-galactosidases

Chapter 1: Literature Review

Proteases

The use of enzymes in biotechnology and therapeutic applications has been drastically increasing. In 2018 the worldwide industrial market for enzymes reached 5.5 billion (Furhan, 2020). Of the enzymes marketed globally, 20% are proteases (Razzaq et al., 2019). In 2019, the global cost of the protease market reached 2.75 billion in the United States. It is projected that the compound annual growth rate for the protease market will increase by 6.1% from 2019-2024 (Matkawala, et al., 2021). Proteases are enzymes that hydrolyze the peptide bond found between amino acids in a polypeptide chain. Ubiquitous in nature, proteases are found in plants, animals, and microorganisms. Within the genome of infectious organisms, proteases account for 1-5% of the genome (Razzaq et al., 2019). In humans, proteases account for more than 2% of the genome and 550 genes (Razzaq et al., 2019; Craik et al., 2011).

Of the 20% of proteases marketed globally, two-thirds are from microorganisms. The use of microbial proteases rather than plant or animal proteases is due to high throughput, less time consumption, less space required for cultivation, cost-effectiveness, and ease to which their genes can be manipulated. Microorganisms grow at relatively rapid rates, decreasing time of labor (Matkawala, et al., 2021). *Bacillus* species are one of the most effective producers of extracellular alkaline proteases. When secreted into the extracellular environment (liquid broth) the protease can be easily purified. Purifying proteases from plants and animals is not as simple and is labor intensive (Razzaq et al., 2019).

Proteases are grouped into clans and families based on sequence similarities (Razzaq et al., 2019). Proteases are also classified based on origin (plant, animal, microorganism), catalytic action, active site residues, pH, molecular weight, charge, and substrate specificity (Matkawala

et al., 2021; Ward, 2011; Razzaq et al., 2019). Based on catalytic activity, proteases are further classified either as exopeptidases or endopeptidases. Exopeptidases hydrolyze at or near the amino (aminopeptidases) or carboxyl (carboxypeptidases) terminal of polypeptide chains (Markawala et al., 2021; Ward 2011). Depending on the size of the fragment cleaved, exoproteases can be further classified such as dipeptide or tripeptide. Microorganisms known to produce aminopeptidases include *Aspergillus oryzae*, *Bacillus licheniformis*, *Botulinum stearothermophilus*, *Escherichia coli*. Microorganisms known to produce carboxypeptidases include to *Aspergillus*, *Penicillium*, *Saccharomyces* species (Ward, 2011).

Endopeptidases hydrolyze internal peptide bonds distant form the amino and carboxyl terminal (Ward, 2011). According to MEROPS (online database for proteases and their inhibitors) endoproteases can be classified into 9 families based on the nature of their active site. The following are residues found in the active site of various proteases: glutamic, serine, asparagine, aspartic, mixed, threonine, unknown (Matkawala et al., 2021). Serine endoproteases account for one-third of the enzymes marketed globally (Razzaq et al., 2019). Within the catalytic site of serine endoproteases, serine residues participate the in the catalytic reaction of hydrolyzing peptide bonds as well as esters and amides. Serine endoproteases function at an optimal pH range of 7-12, with proteases functioning between pH 9-10 known as serine alkaline proteases. Aspartic endoproteases contain a set of aspartic acid residues within their active site and are classified as pepsin/pepsin-like enzymes or rennet/rennet-like enzymes. Microorganisms known to produce pepsin or pepsin-like enzymes include *Aspergillus*, *Penicillium*, *Rhizopus*. *Mucor* and *Endothia* species are known to produce rennet or rennet-like enzymes. Cysteine or thiol endoproteases contain cysteine-histidine within their catalytic site. Organisms such as *Clostridium histolyticum* and *Streptomyces* species produce cysteine endoproteases such as

clostripain and streptopain. Lastly within the active site of endoproteases, divalent cations such as zinc may be found. Metalloendoproteases are thermally stable proteases produced by several microorganisms such as *Bacillus* (Ward, 2011).

Proteases can also be classified as acidic, neutral, or alkaline proteases. Acidic proteases are stable and active at a pH between 3.8-5. With a molecular weight ranging from 30-45 kDa, acidic proteases are most found in fungal species such as *Aspergillus*, *Penicillium*, *Rhizopus*, Mucor, and *Endothia*. Neutral proteases are active at a neutral or weakly acidic or weakly basic pH, pH 5-8. Most neutral proteases are produced by the genus Bacillus. Alkaline proteases are active at a pH between 9-14. Distributed in water and soil alkaline proteases have been isolated from *Bacillus*, *Photorhabdus*, *Salinivibrio*, and *Cryptococcus* species (Razzaq et al., 2019). Many researchers are exploring novel isolates capable of producing alkaline serine proteases stable at high temperatures and pH, exhibit broad substrate specificity, and proteolytic stability (Matkawala et al., 2021).

β-galactosidase

β-galactosidase, commonly referred to as lactase, is an enzyme known to hydrolyze the β-1,4-glycosidic linkage resulting in the production of monosaccharides glucose and galactose. Lactose is an essential disaccharide needed to promote and stimulate the growth of beneficial bacterial in the human gut such as *Lactobacillus* and *Bifidobacterium* species. However, if lactose is unabsorbed, lactose leads to tissue dehydration and the reduction of calcium (Ca^{2+}) absorption. Patients experience excessive diarrhea, cramps, and flatulence (Saqib et al., 2017).

Absorption of lactose requires β-galactosidase. β-galactosidase is present in mammalians during the breast-feeding stage, however, once the breast-feeding stage ceases, the enzymatic activity of β-galactosidase decreases. Lactose is a carbohydrate present in milk. Individuals

lacking or deficient in β-galactosidase result in lactose intolerance and are unable to consume milk or dairy products. Lactose intolerance is estimated to affect 70% of the adult population world-wide. β-galactosidase have been identified in bacteria, yeast, fungi, and plant species. Treating milk and dairy products with β-galactosidase directly offers a solution to lactose intolerance. However, many β-galactosidases have an optimal temperature above 35 °C (Table 1). Medications are also currently available containing β-galactosidase and must be taken prior to the consumption of milk and dairy products (Saqib et al., 2017).

Microorganism Source of β-galactosidase	Optimal temperature (°C)
Bacteria	
Escherichia coli	40
Lactobacillus thermophilus	55
Bacillus circulans	65
Yeast	
Kluyveromyces lactis	30-35
Kluyveromyces fragilis	30-35
Fungal	
Aspergillus niger	55
Aspergillus foetidus	35-45
Aspergillus oryzae	55-60
Neurospora crassa	4

Table 1: Optimal Temperature of β-galactosidases. Optimal temperatures of β-galactosidases isolated from bacteria, yeast, and fungi (Saqib et al., 2017).

Galacto-oligosaccharides (GOS) are also produced from "β-galactosidase by the transglycoslyation activity during the hydrolysis of lactose". GOS are undigested prebiotics that promote a healthy human gut by modifying and promoting the growth of beneficial bacterial. GOS are produced by when the β-galactosidase (E) forms a complex with lactose. A glucose residue is released resulting in the complex enzyme and galactosyl residue. The complex is transferred to an acceptor contain a hydroxyl group (OH-) such as water or a neutrophil-saccharide (Nu) resulting in the formation of GOS (Figure 1). Research is focused on isolating

microorganisms that produce highly efficient β-galactosidase to treat lactose intolerance and improve GOS (Saqib et al., 2017).

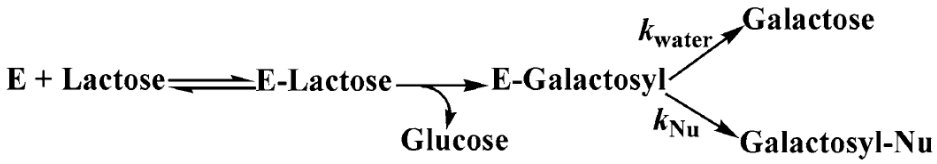

Figure 1: Synthesis of Galacto-Oligosaccharides (GOS). GOS are produced by when the β-galactosidase (E) forms a complex with lactose. A glucose residue is released resulting in the complex enzyme and galactosyl residue. The complex is transferred to an acceptor contain a hydroxyl group (OH-) such as water or a neutrophil-saccharide (Nu) resulting in the formation of GOS (Saqib et al., 2017).

Cold-Adapted Microorganisms

Life on Earth exists at 122 °C in thermal environments and -20 °C in the permafrost. The vast majority of Earth's surface, 80%, is perpetually cold with temperatures below 5 °C. Earth's surface is covered by 70% of oceans, permafrost constituting 20% of terrestrial environments, and 15% polar regions. Within these cold geographical regions, cold-adapted microorganisms known as psychrophilic or psychrotrophic (psychrotolerant) microorganisms exist. Psychrophiles are microorganisms exhibiting optimal growth at 15 °C, a maximum growth temperature of 20 °C, and a minimum growth temperature of 0 °C. Psychrotrophs are microorganisms with an optimal growth temperature ranging between 20 °C and 40 °C, but are capable of survive at 0 °C. Psychrophiles and psychrotrophs (gram-negative, gram-positive, fungi, yeast, and archaea) have been isolated from arctic regions, polar regions, deep sea and glacier soils, glacier ice, permafrost, cold desert soil, sub-Antarctic sediments, sub-glacial water, and alpine regions (Table 2; Joshi & Satyanarayana, 2013). Researchers reported reproduction of cold-adapted

microorganisms can occur at -12 °C, metabolic function at -20 °C, photosynthesis at -17 °C (Furhan, 2020).

Psychrophiles	Isolated From
Azospirillium species	Mountain soil
Bacillus licheniformis	Glacier soil
Clostridum species	Antarctic regions
Colwellia species	Sea ice and sub-Antarctic sediments
Curtobacterium luteum	Glacier soil
Exiguobacterium species	Cold desert soil
Penicillium chrysogenum	Cold marine environment
Pseudomonas species	Deep sea
Serratia species	Coastal water
Vibrio species	Marine water
Xanthomonas maltophilia	Alpine environment

Table 2: Psychrophiles Isolated from Cold Geographic Regions. Microorganisms isolated from cold geographic regions (Joshi & Satyanarayana, 2013).

Cold-adapted microorganisms have adapted to cold harsh environments. To inhibit crystallization, small antifreeze or ice binding-proteins (AFP) are secreted into the extracellular environment to adsorb to ice. AFP arrests the freezing process and lowers the freezing point. Cold-shock proteins are single stranded molecules that bind to nucleic acid and regulate cellular processes such as transcription, translation, protein folding, and the fluidity of cell membranes. Within the membrane of fungi and yeast, polyunsaturated fatty acids are stored to main fluidity at lower temperatures. Cold acclimation proteins maintain cell division and growth and lower temperatures (Furhan, 2020).

In 2011, water samples were collected in the summer months from Uruguay Lake, Collins Bay, and 2 temporary gullies near Uruguayan Antarctic Base on King George Island. Samples were filtered and purified on Luria-Bertani (LB) media. Isolated colonies were then streaked on LB media and minimal milk (MM) agar and grown at 4 °C and 30 °C. Of the 45

isolated colonies obtained, 13 isolates exhibited signs of proteolysis on MM agar at 4 °C and 9 isolates grew at 30 °C without evidence of proteolysis. Using 16S rDNA analysis, sequencing similarities were compared against the NCBI database. Of the 13 isolates exhibiting proteolysis at 4 °C, 9 isolates were identified as *Pseudomonas* species and 4 isolates were identified as *Flavobacterium* species (Martinez-Rosales & Castro-Sowinski, 2011).

To examine the growth rate and temperature, isolates were inoculated in 5 mL LB broth at 4 °C, 18 °C, and 30 °C with constant agitation. Following incubation, the optical density was determined at 620 nm. All 9 isolates of *Pseudomonas* grew at all testing temperatures, however, *Flavobacterium* isolates grew at 4 °C and 18 °C but not at 30 °C. Protease production was determined at the above testing temperatures by inoculating isolates into 5 mL of MM medium and incubating until milk coagulation and proteolytic activity was observed. Proteolytic activity was measured by mixing cell-free supernatant, Azocasein and Tris buffer for 20 minutes at 4 °C and 30 °C. The reaction stopped by adding trichloroacetic acid and the absorbance read at 340 nm. Proteolytic activity was seen at 4 °C and 18 °C for all the isolates. Growth detected at 30 °C but no proteolytic activity measured for the *Pseudomonas* isolates (Martinez-Rosales & Castro-Sowinski, 2011).

Characteristics of Cold-Active Proteases

Cold-adapted microorganisms could source commercially and industrially important novel enzymes with new desirable properties such as enzymatic activity at low temperatures. Unlikely proteases produce from mesophilic organisms, cold-active proteases have evolved in nature. Proteases from mesophilic organism's rate of chemical reaction substantially decreases when subjected to cold temperatures. Cold temperatures increase the compact folding of proteins, therefore, restricting the conformational structure needed for catalysis. The mechanism

enabling enzymatic reactions at cold temperatures is unclear, however cold-active proteases possess characteristic properties lacking in mesophilic proteases.

Cold-active proteases have both a high specific activity (K_{cat}) and high catalytic efficiency (K_{cat}/K_m). K_{cat} is the number of substrate molecules transformed into products by a single enzyme. Michaelis–Menten constant (K_m) is the substrate concentration to which half of the active sites on the enzyme are saturated. K_{cat}/K_m is the affinity to which the substrate is attracted to the enzyme active site (Joshi & Satyanarayana, 2013). Cold adapted trypsin from Atlantic cod exhibited a catalytic efficiency 17 times greater, 2-fold increase in specific activity, and an 8-fold reduction in the Michaelis-Menten constant than trypsin from bovine sources (Fornbacke & Clarsund, 2013). Cold-active proteases also have a lower optimal temperature for enzymatic activity. Proteases isolated from *Penicillium chrysogenum* have an optimal enzymatic activity between 15 °C and 35 °C. When the temperature is decreased to 0 °C, the protease retains 41% of its enzymatic activity. *Pedobacter cryoconitis* proteases have an optimal enzymatic activity at 40 °C, however, enzymatic activity decreases when heated to 50 °C and total inactivation observed at 60 °C (Joshi & Satyanarayana, 2013).

Like other enzymes, each cold-active protease is affected differently by pH, metal ions, and inhibitors. Alkaline proteases isolated from *Stentrophomonas maltophilia* MTCC 7528 have an optimal pH of 10 where maximum protease activity is observed. *Pseudoalteromonas* species NJ276 proteases have an optimal activity of pH 8. Proteases isolated from *Bacillus cereus* species have an optimal pH of 9. Dependent on the enzyme, the effect of pH on proteolysis varies. Protease secreted by *Pseudoalteromonas* species NJ276 are inhibited by Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Fe^{2+}, Pb^{2+}, and Mn^{2+}. Proteases secreted from *Pseudomonas* species strain DY-A is inhibited by Co^{2+}, Cu^{2+}, Zn^{2+}, but is enhanced by Ca^{2+} and Mg^{2+}. Proteolytic activity is also

affected by phenylmethylsulfonylfluoride (PMSF), sodium dodecyl sulfate (SDS), urea, thiourea, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and ethylene glycol tetraacetic acid (EGTA) (Joshi & Satyanarayana, 2013).

Therapeutic and Biotechnology Applications of Proteases

With an increase in the world-wide protease market, proteases have been used in various industries such as detergent, leather, pharmaceutical, food and beverage, waste management, chemical, and the medical industry (Matkawala et al., 2021; Razzaq et al., 2019). Proteinaceous debris and tear films deposit on the surface of contact lens during the normal course of wearing. Proteases from plants and animals such as papain, pancreatin, trypsin, and chymotrypsin have been used remove the debris and tear films. However, allergic reactions and irritation have been reported. Microbial enzymes from *Aspergillus*, *Bacillus*, and *Streptomyces* species have been reported to be an effective contact lens cleanser.

Proteases isolated from *Bacillus* specie 158 has an optimal pH of 7 and optimal temperature of 30 °C. Proteolytic activity reported at pH 5-9 and temperatures 20-60 °C. In an experimental study the transmittance of contact lens was determined using spectroscopic analysis. Prior to the study the transmittance of a contact lens was 98%. After lysozyme was deposited on the surface, the transmittance decreased to 71%. Following treatment with proteases isolated from *Bacillus* specie 158, the transmittance increased to 97% (Pawar et al., 2009). Other proteases such as streptokinase, an extracellular protease produced from hemolytic *Streptococcus* species, have medicinal use. Streptokinase is currently being used to treat acute blockage of arteries, deep vein thrombosis (DVT), and pulmonary embolisms due to its ability to dissolve blood clots. Botulinum toxin is used to treat disorders involving involuntary muscle movements as well as cosmetic use (Ward, 2011). Subtilisin, produced by several *Bacillus* species, has been

developed as ointments, gauze, bandage material used to treat burns, carbuncles, furuncles, and wounds (Razzaq et al., 2019; Matkawala et al., 2021).

To further understand cell development, RNA seq has been combined with cold active proteases to develop a high-resolution atlas of gene expression programs driving kidney development. RNA seq is a powerful technique used to examine the sequence and quantity of RNA in a single cell suspension. High resolution techniques, such as RNA seq, are required to define and characterize cells in heterogeneous mixtures. For instance, tumor cells are composed of vascular cells, fibroblasts, rapidly dividing cancer cells, inert cancer stem cells, and more. Prior to analysis, organs and tissues must be broken down into a single cell suspension which involves incubation at 37 °C with a protease. However, at 37 °C the transcriptional machinery is active and artifactual changes in gene expression are observed. To overcome this challenge cold active proteases have been used to produce a single cell dissociation.

In 2017, kidney samples were collected from 12 postnatal day 1 CD1 mice and minced. Fifty milligrams of minced tissue were added to a protease solution composed CaCl2, DNAse, and a protease isolated from *Bacillus liceniformis* (isolated from the Himalayan glaceries) or a protease solution composed of collagenase, ProNase E, and DNAse. For the cold-active protease procedure, the sample was incubated at 6 °C and triturated for 7 minutes. Trituration is the process of purify compounds containing solid impurities. After incubation the sample was transferred to ice and homogenized using the Miltenyi gentleMACS. Following homogenization, the sample was incubated at 6 °C and tituration repeated for an additional 6 minutes. Single cell suspension confirmed via microscopy. The cells were then transferred to a tube where phosphate buffer (PBS) and fetal bovine serum (FBS) added. Similar process were used to create a single-cell suspension at 37 °C. Digestion buffer was added to minced tissue and heated at 37 °C for 15,

30, and 60 minutes. Digestion stopped by added PBS/FBS. Cell centrifuged, pelleted, resuspended in PBS/FBS, filtered, pelleted, and resuspend a final time for both procedures (Adam et al., 2017).

Analysis of both single-cell suspensions was conducted using Drop-Seq. Based on the results both protocols were able to separate and differentiate cell types such as podocytes, distal tubules, loop of Henle, endothelial cells, stromal cells, and more. As expected, the cell suspension produced at 37 °C exhibited artifactual gene expression changes in the protypical immediate-early genes *Fos* and *Jun*. The *Fos* gene is responsible for regulating the development of cells destined to become part of the skeleton. Using the cold-active protease method gave gene expression of the *Fos* gene 5. However, when incubated at 37 °C the gene expression was 37,540 (15 min), 71,082 (30 min) and 34,786 (60 min). *Fosb* gene showed similar expression patterns: 5 (cold-active protease), 8,032 (15 minutes at 37 °C), 17,344 (30 minutes at 37 °C), and 15,944 (60 minutes °C). Similar pattern was observed for the *Jun* gene. Lastly, global gene expression was observed. After the 15 minutes incubation at 37 °C, 339 genes changed in expression. After 60 minutes incubation at 37 °C, 539 genes changed in expression. Creating a molecular atlas of kidney development is important for providing significant insight into the realm of nephrological diseases (Adam et al., 2017).

Peribacillus

The genus *Bacillus* is an extremely diverse group of microorganisms found with the Firmicute phylum. Organisms within the genus are known to be pathogenic or opportunistic pathogens. Species such as *Bacillus thuringiensis* and *Bacillus velezensis* are agriculturally important while *Bacillus subtilis, Bacillus clausii, Bacillus licheniformis,* and *Bacillus wakoensis* are important to produce antibiotics and enzymes. *Bacillus* species are often classified as Gram-

positive organisms, endospore forming, and aerobic or facultative anaerobes. However, this these characteristics fail to describe the genus as a whole entity. Many species within the genus are bacillus or coccus shaped, motile or non-motile, and non-spore forming. Due to the loose criteria used to assign species that produce spores in aerobic conditions, the *Bacillus* species heterogenous. Many species within the genus don't share phenotypic or biochemical properties within the type species *Bacillus subtilis* (Patel & Gupta, 2020).

Previous studies have used the 16S rRNA gene tree to identified clusters within the *Bacillus* species: *Bacillus sensu sticto*, *Brevibacillus*, *Geobacillus*, *Lysinibacillus*, *Paenibacillus, Alicyclobacillus, Alkalicoccus, Aneurinibacillus, Gracilibacillus, Hydrogenibacillus, Sporosarcina, Ureibacillus, Solibacillus*, and *Virgibacillus*. With the advancement of technology, multiple independent approaches have been used to obtain genome sequences. There is now over >200 named *Bacillus* species that provide a better representation of the genetic diversity of the genus. Patel & Gupta (2020) began to resolved the heterogeneity and polyphyly of the *Bacillus* genus by performing a comprehensive phylogenic and comparative analysis study using the available genome sequences from the *Bacillaceaei* family (Montecillo & Bae, 2022).Additional species within the genus have been reclassified into novel genera: *Peribacillus, Cytobacillus, Mesobacillus, Neobacillus, Metabacillus,* and *Alkalihalobacillus* (Patel & Gupta, 2020; Montecillo & Bae, 2022).

Peribacillus species are Gram-positive or Gram-variable bacilli. Species are either aerobic or facultative anaerobes. All species studied to date, produce endospores under adverse environmental conditions. Species have been isolated from soils, human gut, animal guts, and the Siberian permafrost (*Peribacillus massiliglaciei*). Organisms within the species grow at a range between 3-45 ℃, with an optimum temperature of 25-37 ℃ (Patel & Gupta, 2020).

Peribacillus frigotolerans

In 1967, *Brevibacterium frigoritolerans* was isolated from soil in Morocco. With the advanced in genotyping techniques, the classification and the taxonomic placement of this bacteria has been in question. Liu et al (2020) evaluated the classification on the basis of phenotypic, phylogenetic, and genomic characteristics. It was suggested, the bacteria be placed in the *Bacillus* genus (Liu et al., 2020). However, Patel and Gupta (2020) resolved the heterogeneity and classified the organism as *Peribacillus frigotolerans.* Molecular markers shared by an evolutionarily related group provides a powerful means of delineation of different species. Conserved signature indels (CSIs) found within genes or protein sequences help differentiate between members of *Peribacillus* genus from other *Bacillaceae*. The CSIs are found in HAMP domain-containg protein, phosphor-N-acetylmuramoyl-pentapeptide-transferase, and stage III sporulation protein. For instance, a single amino acid insertion in the HAMP domain containing protein at position 485-535 is found in the *Peribacillus* species but not *Bacillus* species (Montecillo & Bae, 2022).

Chapter II: Protease from a Novel Antarctica Isolate

Introduction

Each year the poultry industry produces a vast amount of recalcitrate or non-biodegradable waste in the form of chicken feathers (Bhari et al., 2020). Based on dry mass, chicken feathers are composed of 80-90% crude protein, 70% amino acids, vitamins, minerals (calcium and phosphorous), and trace elements (Bhari et al., 2020; Peng et al., 2019). Degradation of chicken feathers provides an excellent opportunity to use as a renewable source of protein and amino acids as livestock food and feed supplements (Bhari et al., 2020). However, 90% of chicken feathers is composed of keratin (Kumar, 2021). Keratin is a fibrous structural protein found in epidermal cells and epidermal appendages of vertebrates (skin, nails, chicken feathers). The protein is rich is cysteine residues and disulfide bonds, which contributes to the stability of the molecule and inefficacy of degradation (Peng et al., 2019).

Degradation of chicken feathers has been attempting using high temperatures, pressure hydrolysis, and chemicals (acid, alkali, dimethyl sulfoxide, or dimethyl formamide). These treatments alone or in combination result in poorly digested feathers and poor yield of amino acids (Bhari et al., 2020; Peng et al., 2019). Recent studies have addressed the biodegradation of chicken feathers using keratinases isolated from bacterial and fungal species. Microorganisms known to produce keratinases used to partially degrade chicken feathers include: Bulkholderia, Cryseobacterium, Pseudomonas, Microbacterium, Bacillus, Stentotrophomonas, Chrysoporium, Acremonium, and Aspergillus (Kumar, 2021; Peng et al., 2019). Bacillus licheniformis BBE11-1 and Stentotrophomonas maltophilia BBE11-1 has been documented to hydrolyzed 35.4% and 22.8% of a 50 g/L when incubated at 37 C and 23 C. Data suggests, keratinase found in S. maltophilia has greater enzymatic activity, however, keratinase found in B. licheniformis is more

abundant. Keratinase from both species were co-inoculated with chicken feathers at 30 Cand 80.8% of a 50 g/L culture of chicken feathers was hydrolyzed (Peng et al., 2019).

Due to the high consumption of poultry products, millions of non-biodegradable waste products are produced. Current use of keratinases from microbial species requires cultures to be incubated at 30 C or higher for enzymatic activity. Keratinases isolated from organisms from Antarctica to degrade chicken feathers are unknown. The overreaching focus of my work was to address the following:

1. Can we identify proteases found from isolates obtained from Son of Salt (SOS) Pond located in the McMurdo Ice Shelf in Antarctica?
2. Can we identify the genes encoding the proteases and design primers from a closely related organism?
3. Can we amplify and clone those genes into a plasmid expression system?

In addition to identifying proteases from isolates obtained from Son of Salt (SOS) Pond located in the McMurdo Ice Shelf in Antarctica, we wanted to identify if β-galactosidases were present. β-galactosidases also referred to as lactase, is an enzyme known to hydrolyze the β-1,4-glycosidic linkage resulting in the production of monosaccharides glucose and galactose. Lactose is an essential disaccharide needed to promote and stimulate the growth of beneficial bacterial in the human gut. However, if lactose is unabsorbed, it leads to tissue dehydration and the reduction of calcium (Ca2+) being absorbed. Patients experience excessive diarrhea, cramps, and flatulence. Lactose is a carbohydrate present in milk. Individuals lacking or deficient in β-galactosidases result in lactose intolerance and are unable to consume milk or dairy products. Lactose intolerance is estimated to affect 70% of the adult population world-wide. β-galactosidase have been identified in bacteria, yeast, fungi, and plant species. Treating milk and

dairy products with β-galactosidase directly offers a solution to lactose intolerance (Saqib et al., 2017). Many available β-galactosidases have an optimal temperature above 35 °C and require heating to hydrolyze lactose, which requires additional time and expenses. The goal was to identify β-galactosidases that work at room temperature.

Materials and Methods

Isolate Preparation

Son of Salt (SOS) Pond isolates from a hypersaline pond located in the McMurdo Ice Shelf in Antarctica collected by Dr. Peter Sheridan were struck for isolation on M9-Skim Milk and R2+X-gal agar plates. The cultures were incubated at room temperature (25 °C) for 18-24 hours. Colonies presenting as blue on R2+X-gal agar (β-galactosidase positive) or had a halo on M9-Skim Milk agar (protease positive) were transferred to R2 agar plates. The cultures were then incubated at room temperature (25 °C) for 18-24 hours. Colonies were then transferred to 5 mL of R2 broth for genomic DNA extraction.

Genomic DNA Extraction

SOS genomic DNA extracted using the following protocol. Of a turbid 5 mL R2 culture, 2 mL were centrifuged at 1400Xg for 5 minutes. Supernatant poured off and the cell pellet resuspended in 750 µL of Cell Lysis Buffer [500 mM NaCl, 50 mM EDTA, 1% SDS (w/v), and 50 mM TRIS (pH 8.0)]. Solution then transferred to a sterile 2 mL tube with 250 mg of sterile glass beads (1:1 mix of 0.1 mm and 0.5 mm diameter beads). Approximately 50 µL of a Lysozyme solution (100 mg/mL) added to the tube and incubated for 30 minutes at 37 °C. After the 30-minute incubation, 5 µL of Proteinase K Enzyme solution added and incubated for 30 minutes at 37 °C. The 2 mL tube was then filled to the top with 10 mM Tris and placed in a boiling water bath for 10 minutes. Samples then placed in a bead beater at maximum speed for 2

minutes. Tubes centrifuged for 5 minutes at 3000 rpm. Approximately, 750 µL of supernatant was transferred to two 1.5 mL tubes with 450 µL of isopropanol. The tubes were inverted 50 times and incubate at -20 °C overnight. After the overnight incubation, the tubes were centrifuged at 1400xg for 5 minutes. Supernatant poured off and nucleic acid pellet washed by adding 200 µL of 70% ethanol. The tubes centrifuged for 5 minutes at 1400xg, and supernatant poured off. Nucleic acid pellets dried in a 37 °C water bath for 30 minutes. Pellets were resuspended in 100 µL of 10 mM Tris and the nucleic acid solution was stored in the freezer.

16S rRNA Analysis

Samples were submitted to the Molecular Research CORE Facility (MRCF) at Idaho State University for 16S rRNA analysis with forward primer (8F) and reverse primer (1492R). Once results were obtained, a blast searched was conducted to identify the most closely related fully sequenced genome. Within the genome, we identified the genes encode proteases.

Primer Design

Using the genome of the most closely related species *P. muralis*, we used the genes encoding the desired proteases to design primers. Primers were designed to amplify from the start codon to the stop codon.

Polymerase Chain Reaction (PCR)

Gene segments amplified using the following PCR conditions and MasterMix reagents (Table 3). A reaction temperature of 95 °C for 10 minutes, enabled the initial denaturation of template DNA into single stranded DNA. The following stages were repeated 35 times resulting in the production of millions of copies of the desired gene segment. Denaturation of the template DNA occurred at a reaction temperature of 95 °C for 1 minute. Primers were then able to anneal to the single stranded DNA during the annealing stage following a gradient temperature range of

35-55 °C for 1 minute. Due to using a closely related genome, during gene amplification we used a gradient on the thermocycler in case mismatches were present. The reaction temperature was then increased to 72 °C for 5 minutes, providing an optimum temperature for DNA polymerase to extend the new DNA strand from the primers. After the 35 cycles were completed, a final extension staged occurred at 72 °C for 10 minutes. PCR products were then run on an agarose check gel to determine the ideal annealing temperature of 50 °C.

Master Mix Solution	Volume (50 µL)
10 X Buffer	5
Nucleic acid water	29
dNTP	8
Forward Primer	2.5
Reverse Primer	2.5
Vent Polymerase	0.5
DNA	2

Table 3: Master Mix Reagents and Volumes. Master Mix reagents and volumes used to amplify gene of interest.

Amplicon Purification

The PCR amplicon product (25 µL) was mixed with 6X loading dye (5 µL) then added to an 1% agarose gel. After 30-45 minutes at 100 V, bands were extracted with a scalpel under UV light. Excised bands added to pre-weighed sterile 1.5 mL tubes. The weight of the tubes with gel was determined, and the weight of gel was calculated. DNA bands purified from gel using GeneJET PCR Purification Kit (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0012662_GeneJET_PCR_Purification_UG.pdf). Following, DNA purification, an agarose check gel was performed. as stated above.

aLICactor

The aLICator Ligation Independent Cloning (LIC) and Expression System permits expression of proteins that are toxic to *E. coli* cells. The aLICator system ensures 95% cloning efficiencies and eliminates the use of restriction enzymes and ligation reactions. The aLICator cloning system recommends two different host cells, one for sequencing and another for expression. During the first cloning procedure, XL1-Blue cells are utilized for transformation. Once the recombinant plasmid DNA has been constructed and sequenced, plasmid DNA will be transformed into competent *E. coli* strain BL21(DE3) which contains the T7 RNA polymerase under control of IPTG (Scientific, T. 2013).

Within the pLATE expression vectors, expression of the gene of interest is controlled by a bacteriophage T7 promotor recognized specifically by T7 RNA polymerase. The expression host strain, BL21 (DE3), must be utilized because it encodes an inducible promoter to control T7 RNA polymerase expression required for target gene expression. Within *E.* coli, a DNA region known as the lac operon contains three genes (lac Z, lac Y, lac A). Upstream from the lac operon, a promoter is present and is the site where RNA polymerase begins transcription. When lactose is absent and a repressor binds to the lac operon, transcription is inhibited. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is a chemical reagent that mimics the natural inducer, allolactose. When present IPTG will bind to the repressor resulting in a confirmational change and a decrease in affinity of the repressor to bind to the operon sequence. This results in T7 RNA polymerase binding to the T7 promoter and initiating gene transcription (Figure 2; Buckingham, 2019). The pLATE vectors contain supplementary elements to reduce basal T7 RNA polymerase expression (Table 4; Scientific, T. 2013).

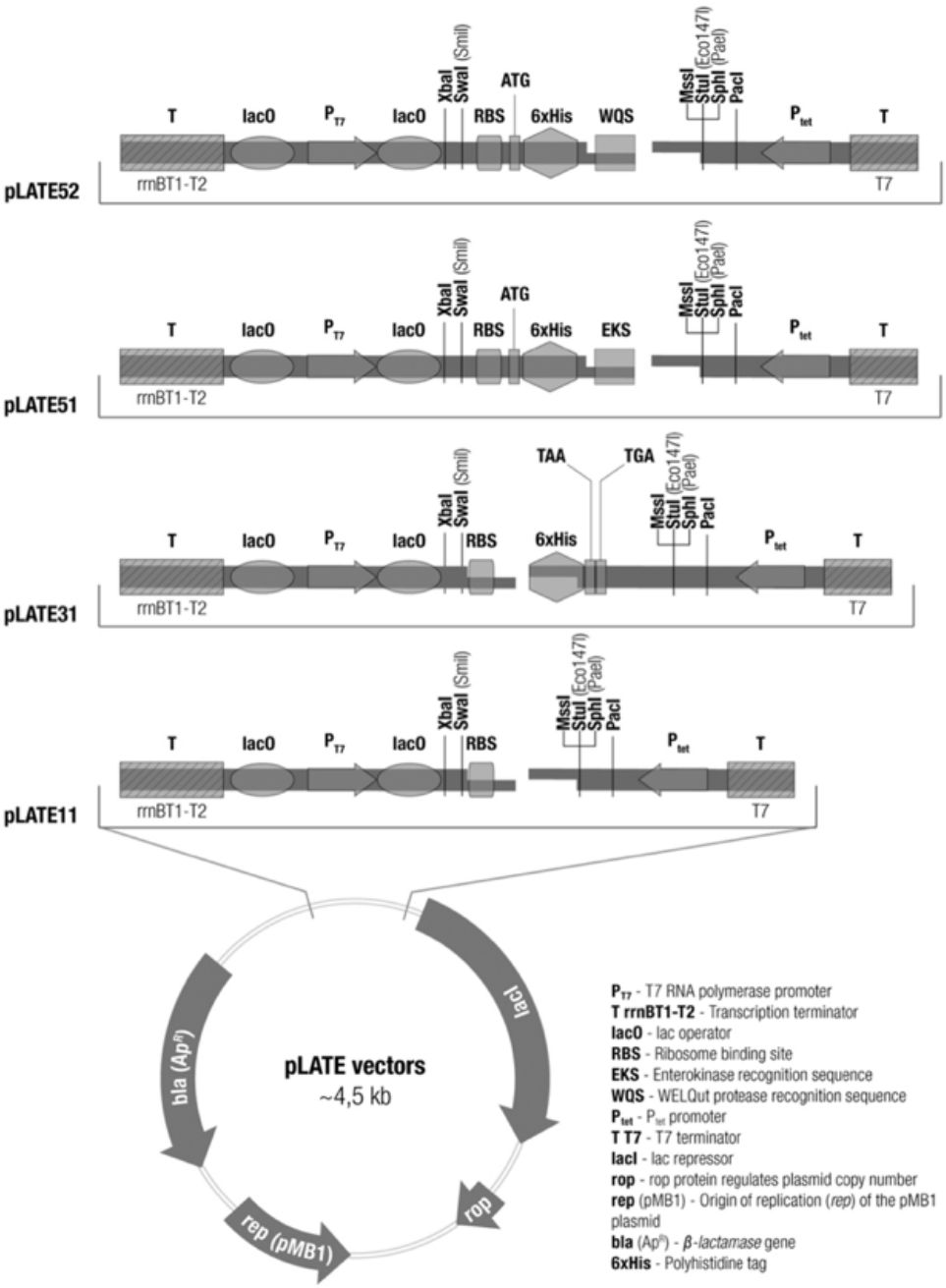

Figure 2: pLATE Vector Design. Using the aLICator Cloning System host cell BL21 (DE3) was utilized. Gene of interest is under control of a strong bacteriophage T7 promoter specifically recognized by T7 RNA polymerase.T7 RNA polymerase in BL21 (DE3) host cells is under control of an inducible promoter. IPTG must be added to transcribe T7 RNA polymerase, which can then be used to transcribe gene of interest (Scientific, T. 2013).

pLATE Elements	Function
Two lac operators flanking T7 promoter	Binding sites for lac repressor
rrnBBT1-T2 Terminator	Prevent basal gene expression due to vector derived promoter-like elements
Tet promoter (P_{tet})	Operates in opposing direction to the T7 promoter, inhibits basal expression
Enterokinase recognition site or WELQut protease recognition site	Enterokinase or a novel WELQut protease can remove the amino terminal histidine tag following protein purification
ATG	Start codon
TAA, TGA	Stop codons

Table 4: Elements of pLATE Cloning Vector and Function. Within the pLATE vector elements are present which aids in optimizing expression (Scientific, T. 2013).

Generating 5' and 3' overhangs

PCR amplicons generated by using the designed aLICactor primers were purified by gel extraction using GeneJet Purification Kit. Using the aLICactor LIC cloning system specific 14-21 nucleotide single stranded 5' and 3' overhangs are generated using T4 DNA polymerase (Table 5). T4 DNA polymerase has 5'-3' polymerase activity and 3'-5' exonuclease activity. Only deoxyguanosine triphosphate (dGTP) was included in the reaction. Once the 5'-3' polymerase activity incorporates dGTP in the complementary strand, both the 5'-3' polymerase activity and the 3'-5' exonuclease activity reach equilibrium. Reagents (Table 5) were added to a PCR tube and incubated for 5 minutes at room temperature to generate the overhangs. To stop the reaction, 0.6 μL of EDTA was added. The annealing reaction occurred by adding the corresponding vector to each tube (Table 6). Reactions were incubated at room temperature for a minimum of 5 minutes, or a maximum of 2 hours (Scientific, T. 2013).

Component	Volume
5X LIC Buffer	2 µL
Purified PCR Product	5 µL
Nuclease-free water	2 µL
T4 DNA polymerase	1 µL

Table 5: Reaction Mixture Used to Generate 5' and 3' Overhangs. pLATE vectors require PCR amplicons to have overhangs present. T4 DNA polymerase was utilized to added 5' and 3' overhangs to PCR amplicons (Scientific, T. 2013).

PCR Product	Vector
No His Tag	pLATE 11 Vector
C-term His Tag	pLATE 31 Vector
N-term His Tag	pLATE 52 Vector

Table 6: Vectors Corresponding to Each PCR Product. Corresponding vectors to the PCR amplicons (No histidine tag, C-terminal histidine tag, and N-terminal histidine tag) were utilized to ligated PCR amplicons into vectors (Scientific, T. 2013).

Two chemical transformations kits were utilized: Zymo Kit and Bacterial TransformAid Kit.

https://files.zymoresearch.com/protocols/_t3001_t3002_mix_go_e._coli_transformation_kit_buffer_set.pdf

https://tools.thermofisher.com/content/sfs/manuals/MAN0012725_TransformAid_Bacterial_Transformation_UG.pdf

Zymo Kit

One day prior to cloning, 25 µL competent cells (DH5α and XL1-BLIE) were inoculated into 5 mL of LB and incubated at 37 °C overnight. The following day 1.5 mL of the turbid competent culture was added to 3 tubes and centrifuged for 5 minutes at 8000 rpm. Supernatant was decanted and 500 µL of wash buffer solution (250 µL wash buffer + 250 µL dilution buffer) added. Samples centrifuged for 5 minutes at 8000 rpm and supernatant poured off.

Approximately 200 µL competent buffer (100 µL competent buffer + 100 dilution buffer) added to the pellet and gently resuspended. The cloning reaction (10 µL) was added to the competent buffer mixed with the pellet and incubated on ice for 30 minutes. The sample was heat shocked at 42 °C for 30 seconds and immediately placed back on ice. LB (800 µL) was added to each tube and incubated at 37 °C for 60 minutes. Approximately 100 µL of each sample was then plated on several LB-ampicillin plates. Plates were incubated overnight at 37 °C. Colonies observed the following day.

TransformAid Bacterial Transformation

One day prior to cloning, 25 µL of competent cells (DH5α) were inoculated into 2 mL of C-media and incubated at 37 °C overnight. Day of cloning reaction the following was incubated at 37 °C for 20 minutes before use: 1.5 mL of C-media, LB-ampicillin plates, and 5 mL LB broth. T solution A and T solution B were thawed at room temperature before use. Once thawed, 250 µL each solution combined into a tube and kept on ice. Of the overnight bacterial culture, 150 µL was added to 1.5 mL of prewarmed C-media. The culture was incubated at 37 °C for 20 minutes on a rotary shaker. The sample was centrifuged for 2 minutes at 1400 × g, and the supernatant discarded. Cells resuspended in 300 µL of T solution (A + B). The sample was then incubated on ice for 5 minutes and centrifuged for 2 minutes at 1400 × g. The supernatant was discarded, and the pellet gently resuspended in 120 µL of T solution. The sample was then incubated on ice for 5 minutes. In a new 1.5 mL tube, 5 µL of cloning reaction was added and incubated on ice for 2 minutes. After the incubation period, 50 µL of the cells were added to the cloning reaction. The reaction was incubated on ice for an additional 5 minutes. An additional 500 µL of prewarm LB broth was added to each tube. From the reaction, 100 µL was plated on several LB-ampicillin plates and incubated overnight at 37 °C. From the reaction, 100 µL was

plated on several LB- ampicillin plates. Plates incubated overnight at 37 °C. Colonies observed the following day.

Plasmid isolation

Plasmid DNA isolated using SV Promega Kit (https://www.promega.com/products/nucleic-acid-extraction/plasmid-purification/wizard-plus-sv-minipreps-dna-purification-systems/?catNum=A1330). After plasmid DNA was isolated, sample left on benchtop overnight to evaporate residual ethanol used during the washing process. Samples then ran on an agarose gel and visualized using UV.

Plasmid sequencing

Plasmids sequenced at the Molecular Research Core Facility using LIC Forward and Reverse Sequencing primers (Table 7; Figure 3; Figure 4; Figure 5).

Primer Name	Sequences
LIC Forward Sequencing primer, 20-mer	5'-TAATACGACTCACTATAGGG-3'
LIC Reverse Sequencing primer, 24-mer	5'-GAGCGGATAACAATTTCACACAGG-3'

Table 7: LIC Sequencing Primers. pLATE plasmids with insert ligated, sequenced using LIC Forward and Reverse (Scientific, T. 2013).

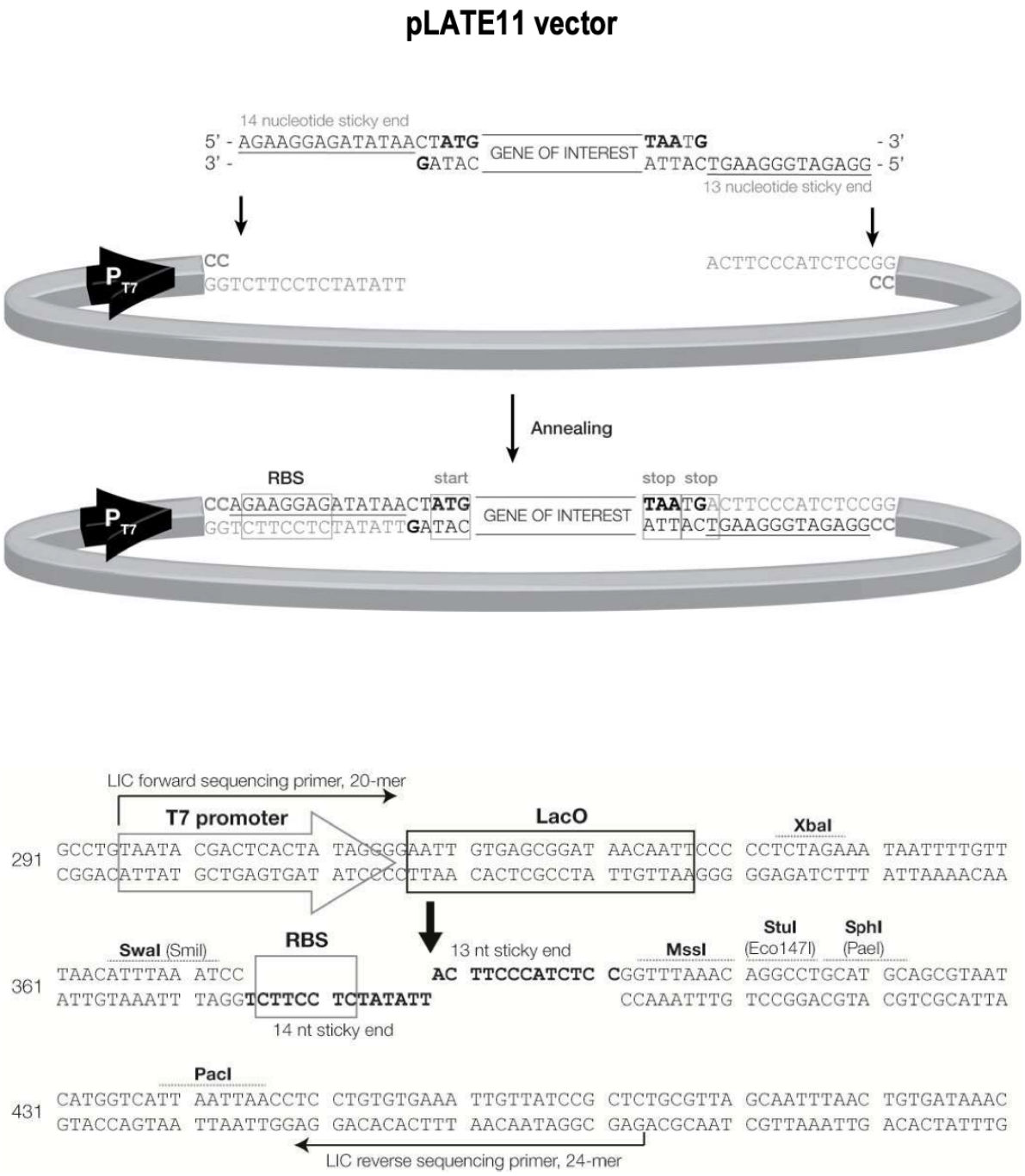

Figure 3: Sequencing of pLATE 11 Vector. PCR amplicons with no histidine tag ligated into pLATE11 vector (Scientific, T. 2013).

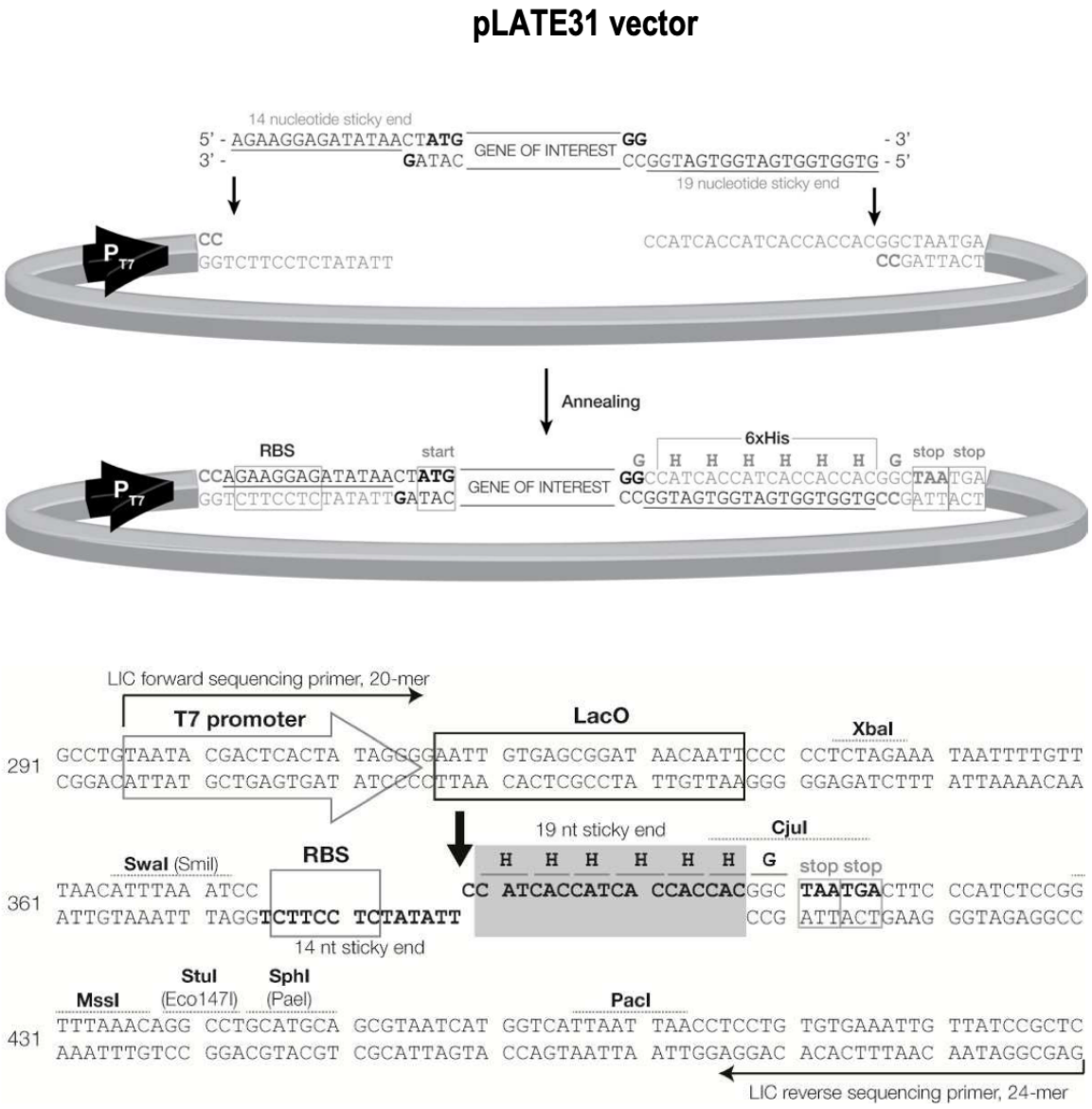

Figure 4: Sequencing of pLATE31 Vector. PCR amplicons with C-terminal histidine tag ligated into pLATE52 vector (Scientific, T. 2013).

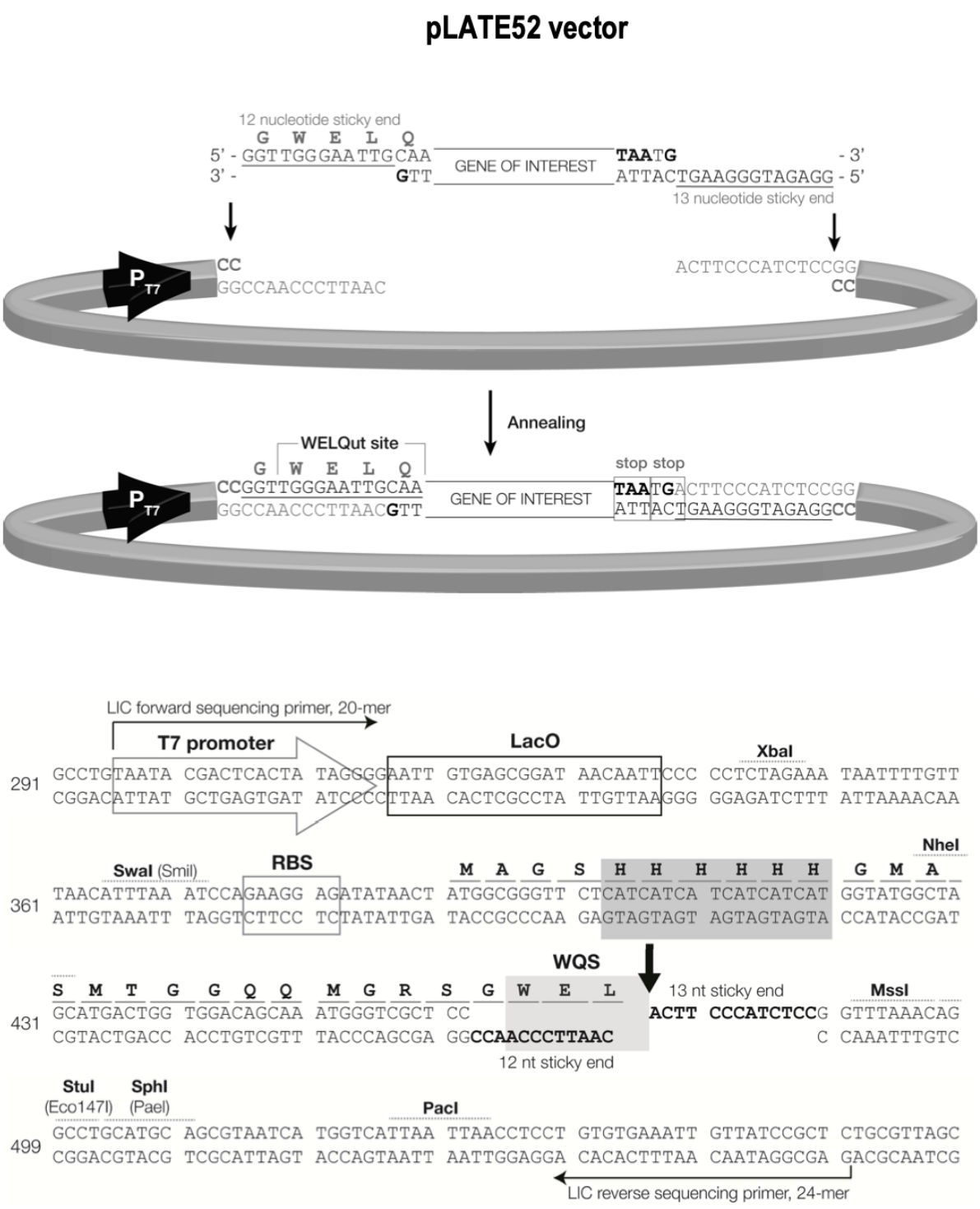

Figure 5: Sequencing of pLATE52 Vector. PCR amplicons with N-terminal histidine tag ligated into pLATE52 vector (Scientific, T. 2013).

After sequences were obtained for the Molecular Research Core Facility and gene target identified, the plasmid was transformed into the expression host strain BL21 (DE3) following the protocol from the TransformAid Bacterial Transformation kit.

Expression Experiments

Expression

After transformations into expression host strain BL21 (DE3), colonies were inoculated into 5 mL LB-ampicillin broth and incubated at 37 °C overnight. Two sterile 50 mL LB-ampicillin broths were inoculated with 500 µL of turbid 5 mL cultures. Cultures were incubated at 37 °C overnight and the following day one culture was induced with 250 µL of 100 mM IPTG. Both cultures (induced and uninduced) were incubated at 37 °C overnight. Cultures were then transferred to 50 mL Oakridge tubes and centrifuged for 30 minutes at 8000 rpm. The supernatant was transferred to a sterile 50 mL falcon tube and stored at 4 °C. Pellets were resuspended in 2 mL of 10 mM Tris containing 20 µL of PMSF. Samples transferred to a 2 mL tube with 250 mg of sterile glass beads (1:1 mix of 0.1 mm and 0.5 mm diameter beads). Samples then placed in a bead beater at maximum speed for 2 minutes. Tubes centrifuged for 5 minutes at 3000 rpm. Supernatant (lysate) transferred to sterile screw cap tube and placed at 4 °C. Following day 25 µL lysate+ 5 µL of 6X loading dye were ran on a 10% polyacrylamide gel and stained with RSS.

Rapid Nickel Resin Purification

In a 2 mL screw cap tube, 200 µL of nickel resin and 100 µL of uninduced or induced C-terminal and N-terminal lysate was added. The sample was then placed horizontally on a rotary shaker for 60 minutes. The tube was then removed from the rotary shaker and placed in an Eppendorf rack for 30 minutes to allow the resin to settle to the bottom. The supernatant was

drawn off and placed in a 2 mL snap cap tube. To elute the proteins of interest from the nickel resin beads, 200 µL of 200 mM imidazole was added to the 2 mL screw cap tube. Sample was placed horizontally on a rotary shaker for 10 minutes. The tube was then removed from the rotary shaker and placed in an Eppendorf rack for 30 minutes to allow the resin to settle to the bottom. The elute was drawn off and placed in a 2 mL snap cap tube. The sample (control), supernatant, and eluate was analyzed via PAGE followed by RSS.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was done by using 10% acrylamide gels to visualize the native structure of proteins found within the spent media. Fisher BioReagent EZ-Run Prestained Protein Marker (2.5 µL) was used to size any resulting bands from the spent media. 25 µL of sample (spent media, spent/filtered media, or spent/filtered/concentrated media) was mixed with 5 µL of 6X loading dye, loaded onto the polyacrylamide gel, and electrophoresed for 90 minutes at 120 volts. Rapid Silver Staining (RSS) protocol was used to determine the location and size of proteins within the gel.

A 10% polyacrylamide gel was placed in a plastic container and 100 mL of fixing solution added and agitated for 10 minutes. Fixing solution poured off and 50 mL distilled (DI) water was added before agitating for 5 minutes. DI water was decanted, and an additional 50 mL DI water added and agitated for 5 minutes. 50 mL of sodium thiosulfate ($Na_2S_2O_3$) added for 1 minute and immediately washed twice with 50 mL of DI water for 20 seconds. 50 mL silver nitrate solution added and agitated for 10 minutes. Silver nitrate solution poured off and 25 mL thiosulfate added for 20 seconds. The $Na_2S_2O_3$ solution was poured off; an additional 50 mL of $Na_2S_2O_3$ solution added. When the desired band intensity was reached 2.5 mL of 2.3 M citrate

solution added for 10 minutes to stop the reaction. Citrate solution poured off and gel washed twice with 50 mL of DI water for 10 minutes. Gel stored in a whirl pack bag.

Media/Solutions

LB broth consisted of 10 g Tryptophan, 10 g Sodium chloride, 5 g Bacterial yeast in 1.0 liter of distilled water.

LB agar plates consisted of 10 g Tryptophan, 10 g Sodium chloride, 5 g Bacterial yeast, 20 grams agar in 1.0 liter of distilled water.

100 mM Phenylmethylsulfonyl fluoride (PMSF) produced by adding 0.871g of PMSF to 1 mL of isopropanol. Solution was stored in a 50 mL falcon tube covered in foil at 4 C.

Ampicillin solution made by adding 1 g ampicillin salt to 50 mL DI water. Solution was filtered through 0.22 μM sterile filter. Solution was stored in a 50 mL falcon tube covered in foil at 4 °C. For 1.0-liter LB-ampicillin broth or 1.0-liter LB-ampicillin Agar, 5 mL of ampicillin solution was added. For 50 mL LB broth, 500 μL of ampicillin solution was added. For 5 mL LB-ampicillin broth, 50 μL of ampicillin solution was added.

100 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) made by adding 119.16 mg of IPTG in 50 mL of diH20 and filtering through a 0.22 μM filter. Solution was stored in a 50 mL falcon tube covered in foil at 4 °C. For 1.0-liter LAXI agar, 2.5 mL of 100 mM IPTG solution added prior to pouring plates. For 5 mL LAXI broth, 25 μL of 100 mM IPTG solution added.

5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-gal) solution was made by adding 1 g of X-Gal in 50 mL of N,N-dimethyl formamide. The solution was stored in a 50 mL falcon tube covered in foil at -20 °C. For 1.0-liter LAX or LAXI agar, 2.5 mL X-gal solution added prior to pouring plates.

50% glycerol stocks were made by adding 80 mL glycerol and 40 mL 10 mM Tris buffer. Sample heated until glycerol dissolved. Aliquoted 750 µL of solution to two mL screw cap tubes. Used to prepare glycerol stocks of cultures.

50X TAE electrophoresis buffer was made by adding 242 g of Tris base, 57.1 mL glacial acetic acid, 37.2 g Na_2EDTA. Reagents diluted to 1 liter with DI water.

1X TAE electrophoresis buffer made by adding 200 mL of 50X TAE electrophoresis buffer and diluted to 1 liter with DI water.

5X Electrophoresis buffer was made by adding 15.1 g Tris base and 72 g of glycine. Reagents diluted to 1 liter with DI water.

1X Electrophoresis buffer was made by adding 200 mL of 5X electrophoresis buffer and diluted to 1 L with DI water.

1% Agarose Gel and Conditions

Rapid Silver Staining Reagents

Formaldehyde Fixing Solution was made using 400 mL methanol, 1 mL of 37% formaldehyde, and diluted to 1 L with diH_2O. The solution was stored at room temperature.

Sodium thiosulfate solution was prepared using 0.2 g sodium thiosulfate diluted to 1 L with diH_2O. The solution was stored at room temperature.

Silver nitrate was made using 1 g silver nitration diluted to 1 L with diH_2O. The solution was stored at room temperature.

The Thiosulfate Developing Solution combined 30 g sodium carbonate, 4 mg sodium thiosulfate, and diluted to 1 L with diH_2O. The solution was stored at room temperature.

2.3 M Citric Acid Solution

Citric acid solution prepared by adding 44.19 g citric acid and diluting it to 100 mL with DI water.

Results

SOS isolates were struck on M9-Skim Milk and R2+X-gal agar plates (Table 8).

Isolate Name	M9-Skim Milk Agar	R2+ X-gal Agar
SOS 1C-A1	No halo around colonies	No blue colonies
SOS 1C-A2	No halo around colonies	No blue colonies
SOS 1C-A3	Halo around colonies	Blue colonies
SOS 1C-B1	No halo around colonies	Blue colonies
SOS 1C-B2	No halo around colonies	No blue colonies
SOS 1C-C	No halo around colonies	No blue colonies
SOS 1C-E	No halo around colonies	Blue colonies
SOS 2A-C	No halo around colonies	No blue colonies
SOS 2A-D	No halo around colonies	No blue colonies
SOS 2A-F	No halo around colonies	No blue colonies
SOS 2A-G	No halo around colonies	Blue colonies
SOS 2B-B	No halo around colonies	No blue colonies
SOS 2B-C	No halo around colonies	No blue colonies
SOS 2B-E	No halo around colonies	No blue colonies
SOS 2B-F1	No halo around colonies	No blue colonies
SOS 2B-F2	No halo around colonies	Light blue colonies
SOS 2C-B	No halo around colonies	Blue colonies
SOS 2C-C	No halo around colonies	No blue colonies
SOS 2C-F1	No halo around colonies	Blue colonies
SOS 2C-F2	No halo around colonies	Blue colonies
SOS 2C-G	No halo around colonies	No blue colonies
SOS 2C-I	No halo around colonies	No blue colonies
SOS 2C-J	No halo around colonies	No blue colonies

Table 8: SOS Colony Appearance on M9-Skim Milk and R2+ X-gal Agar Plates. M9-Skim milk agar enables the identification of proteases present. A zone of clearing surround a colony is positive for proteases being present. R2+X-gal agar enables the identification of β-galactosidase.

Blue colonies on R2+X-gal and protease positive colonies on M9-Skim Milk agar plates were transferred to R2 agar plates (Table 9).

Isolate Name	Isolate Renamed	Colony Morphonology on R2 Agar Plates
SOS 1C-A3	4	White, punctiform
SOS 1C-B1	5	No growth
SOS 1C-E	8	White, punctiform
SOS 2A-G	6	White, small, smooth edges, flat
SOS 2B-F2	7	No growth
SOS 2C-B	1	No individual colonies, growth yellow/orange
SOS 2C-F1	2	White, small, irregular edges
SOS 2C-F2	3	White, small, irregular edges

Table 9: SOS Isolates on R2 Agar Plates.

Following genomic DNA extraction, samples were analyzing on an agarose gel and visualized under UV light (Figure 6).

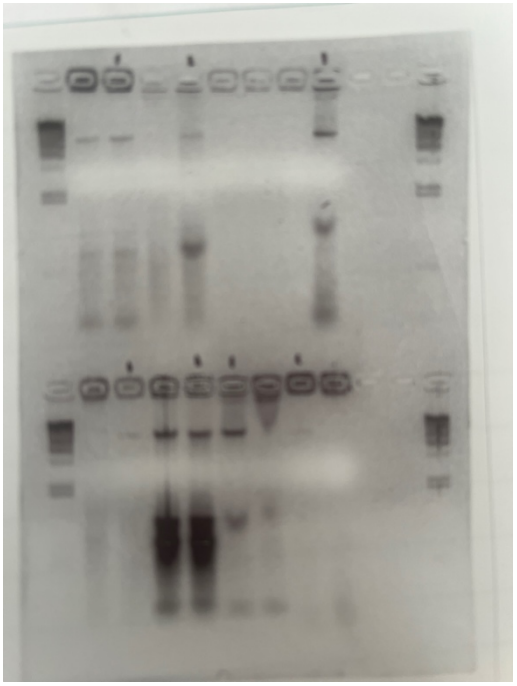

Figure 6: Genomic DNA of SOS isolates. Each sample was run in duplicate. From left to right (top row): protein ladder, 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B. From left to right (bottom row): protein ladder, 5A, 5B, 6A, 6B, 7A, 7B, 8A, 8B.

A 16S rRNA analysis was performed on the following isolates 1B, 2B, 4B, 5B, 6B, 7A, and 8A. Once data was obtained a Basic Local Alignment Search Tool (BLAST) searched was conducted to identify the most closely related organism *Peribacillus frigoritolerans*. However, at the time of the blast search, the genome of *P. frigoritolerans* was incomplete. We were able to identify a closely related organism *Peribacillus muralis*, whose genome was fully sequenced. We searched the genome to identify possible proteases. Following are the amino acid sequences for Serine Protease (SP1), Protease 5 (P5), and Zinc Protease 2 (ZnP2):

Serine Protease Protein Sequence (332 amino acids)

MTREKRLIPYKVLEVMETVKEKIPEGIELVKAPAVWEKSNYGEGVVVAVIDTGIDKEHP
DLKERIIGGKDFTNTGDFQDDNGHGTHVCGTILASINKVGVVGVAPKASILALKALNGQ
GQGEIDWINGALEYAINWQGPNEETVSVISLSLGGPPDEAEHKLIQRALKKDILVVCAAG
NSGDGRHDTDELDYPGAYPEVVEVGAVDLSRNLADFSNTNDEIDLVAPGVDILSTYPGN
KYARLSGTSMATPHVSGAAALLKVIAEQDFGRKLTEAELYAQLVKSTEDLGISKKAQG
NGLLNLTIADQRAEKSIKITIESENLKLPSKSVVMEY*

Protease 5 Protein Sequence (353 amino acids)

MHKKDCSKSVCQCFIVPNYILENLALNEVESARISLAKSRQMRRRRIFKEYDINGVVALT
DAGSGRHGESARHVFDCKGGTKLRVNEARKEGDRSSGDPVVNQAYDYSGVVRDYFKD
VLHRNSMDNEGLDLILNVHYGNKFTNAYWDGDEMVFGDGDGVIFSNFANSLDVIGHEL
THGVTQFTSGLEYEGQSGALNEHLSDVFGVTIKQYHLKQTAGDADWLIGADIMGPTLR
GQALRSMKAPGTAFDNKLMGKDMQPDHMRNFYKGERDNHGVHINSGIPNKAFYLVS
MEIGTDKAALIWFDAMQNLFATANFNQFVQVVIKAAQKLADGGKVPHAAIKTIEEAFK
AVGLPE*

Zinc Protease 2 Protein Sequence (426 amino acids)

MSIASDTIMEKSGYRLHVVRTDKYKTNTLVLKMKAPLTKEKVTYRALVPYVLQSNTSK
YPTTPELRSYLDELYGAGFYVDVAKKGEYQIVSFTIDIVNEKFLSDSTPLLEKAFGLLSEV
IFHPKKNGEAFDPKTVTNEIRSLKQRLQSISDDKMRYSATRLVEEMCKTEPYALEASGNL
EDLKTITPESLFTYYQKMLAEDEIDLYVIGDINESEVEALADKYVTLKARVPVRLPRNTD
KEVEKEKEVIENSDVKQGKLNIGYRTHIAYGDPDYYALQLFNGIFGGFSHSKLFINVREK
ASLAYYAASRLESHKGLLMVMAGIENANYKQALEIIHAQMNEMKQGNFSEAELEQTKA
VVRNQLLETIDVSRGLVEILYHNVISGQNISLDDWFAETERTTKEEIVAVGQKIQLDTIYF
LTEAGVQE*

We used the genes encoding the desired proteins to design primers to amplify the target genes. The resulting primers were used to amplify the identified genes from the start codon to the stop codon. Forward primer and reverse complement primers were generated for SP1, P5, and ZnP2 genes:

Serine Protease Gene Sequence (999 nucleotides)

Forward Primer
ATGACAAGGGAAAAAAGATTAATACCTTACAAAGTTCTTGAAGTGATGGAAACGG
TGAAAGAAAAGATCCCAGAGGGTATCGAGCTTGTTAAAGCCCCGGCAGTATGGGAA
AAAAGCAATTATGGTGAGGGTGTTGTAGTCGCCGTCATCGATACAGGCATCGATAA
AGAGCATCCAGACCTCAAAGAGAGAATCATTGGCGGAAAGGATTTTACCAATACCG
GCGATTTTCAAGATGATAACGGGCATGGAACGCATGTATGCGGGACTATATTGGCTT
CCATTAATAAGGTTGGTGTAGTGGGAGTGGCTCCAAAAGCCAGCATCTTGGCGTTGA
AGGCATTAAATGGTCAGGGCCAAGGCGAGATAGATTGGATCAATGGGGCTTTGGAG
TATGCAATCAATTGGCAGGGGCCGAATGAAGAAACAGTTTCAGTCATTTCACTGTCA
CTTGGAGGGCCGCCGGATGAGGCGGAACATAAGCTGATTCAAAGGGCTCTTAAGAA
AGACATTCTTGTTGTTTGCGCGGCTGGGAACAGTGGGGATGGGCGTCATGATACCGA
TGAATTGGATTATCCAGGTGCGTATCCAGAGGTAGTGGAAGTCGGGGCTGTGGATTT
AAGCAGAAATTTAGCGGATTTTTCAAATACGAATGATGAAATTGACTTGGTTGCGCC
GGGGGTCGATATACTCTCTACGTATCCAGGTAATAAATATGCCAGACTAAGCGGAA
CATCAATGGCAACACCGCATGTAAGCGGTGCAGCAGCTCTCTTGAAAGTGATTGCCG
AACAGGACTTTGGCCGCAAGCTGACTGAAGCTGAACTGTATGCACAGTTAGTGAAA
AGTACGGAAGATCTGGGAATTAGCAAAAAAGCGCAAGGCAATGGGCTGCTCAACTT
AACCATTGCCGACCAAAGAGCTGAAAAATCAATCAAGATTACTATAGAGTCAGAGA
ACCTTAAATTA**CCGAGTAAATCAGTGGTCATGGAATATTAA**
Reverse Complement Primer

Protease 5 Gene Sequence (1062 nucleotides)

Forward Primer
ATGCACAAAAAGGATTGTTCCAAATCGGTGTGCCAGTGTTTCATAGTACCTAATTA
TATCTTAGAAAATTTGGCCCTGAATGAGGTGGAATCGGCAAGGATCAGTTTAGCGA
AAAGCCGGCAAATGCGAAGGCGCCGAATCTTTAAGGAATATGATATCAATGGTGTC
GTCGCTTTAACCGATGCTGGCTCAGGCAGGCATGGTGAGTCGGCACGGCATGTATTT
GATTGCAAAGGTGGAACGAAGCTCAGGGTGAATGAGGCAAGGAAAGAAGGAGACC
GATCAAGCGGTGATCCAGTTGTTAACCAAGCATATGATTATAGTGGAGTAGTTCGTG
ATTATTTCAAGGATGTCCTGCACCGTAATTCAATGGATAACGAAGGCTTGGATCTCA
TTCTGAACGTCCATTATGGCAATAAATTCACGAATGCCTACTGGGATGGCGATGAAA
TGGTTTTCGGAGATGGCGATGGGGTCATTTTTAGCAATTTTGCCAACTCACTCGATGT
GATTGGGCATGAGCTTACACATGGGGTGACGCAGTTCACGAGTGGTTTGGAGTATG
AAGGTCAATCAGGGGCTTTGAATGAACATCTGTCAGATGTGTTTGGAGTGACCATAA

AGCAATATCACTTAAAGCAGACGGCTGGAGATGCCGATTGGCTGATTGGTGCCGAT
ATAATGGGACCCACACTGAGGGGACAGGCTCTTCGTTCCATGAAAGCACCTGGTAC
GGCATTTGATAATAAATTGATGGGGAAGGATATGCAGCCTGATCATATGAGAAACT
TCTATAAAGGCGAGCGGGATAATCATGGAGTCCATATCAACAGCGGGATACCAAAC
AAGGCCTTTTATCTTGTATCAATGGAAATCGGAACGGACAAAGCGGCCTTGATTTGG
TTTGATGCGATGCAGAATTTATTTGCGACAGCGAATTTCAATCAATTCGTACAAGTT
GTCATCAAGGCTGCCCAAAAGCTTGCGGATGGTGGCAAGGTGCCTCATGCAGCGAT
CAAAACGATTGAAGAA**GCGTTTAAAGCTGTCGGGCTGCCTGAATAG**

Reverse Complement Primer

Zinc Protease 2 Gene sequence (1281 nucleotides)

Forward Primer

ATGTCTATCGCTTCCGATACAATAATGGAAAAAAGCGGATACAGGCTTCATGTGG
TCCGTACGGATAAATATAAAACGAATACGCTCGTGCTGAAGATGAAAGCGCCATTG
ACGAAAGAAAAAGTTACATATCGCGCGTTGGTGCCATATGTGTTACAGAGCAATAC
GAGTAAATATCCTACGACTCCTGAGCTTAGATCTTATCTTGATGAATTATACGGCGC
GGGTTTTTATGTCGATGTCGCAAAAAAAGGAGAATATCAAATCGTTAGTTTCACCAT
CGATATCGTCAATGAAAAATTCCTTAGTGATTCAACGCCGCTTCTAGAAAAAGCTTT
TGGGTTATTATCTGAGGTGATCTTTCATCCGAAAAAGAATGGCGAGGCCTTTGATCC
TAAAACGGTGACCAATGAAATCCGTTCCTTGAAACAGCGACTTCAATCCATTTCCGA
TGATAAAATGCGCTATTCGGCTACAAGGCTTGTCGAGGAAATGTGCAAAACCGAAC
CATATGCCCTTGAAGCAAGCGGAAACCTAGAGGACTTGAAAACGATAACGCCGGAG
TCCCTTTTCACTTATTATCAAAAAATGCTGGCTGAAGATGAGATCGATTTGTATGTG
ATCGGCGACATCAATGAATCGGAGGTCGAGGCTTTAGCTGACAAATATGTGACCTTA
AAGGCTCGGGTACCGGTACGTCTGCCAAGGAATACGGACAAGGAAGTGGAGAAGG
AAAAGGAAGTCATCGAAAACTCGGATGTAAAACAAGGGAAATTGAATATCGGCTAC
CGTACCCATATTGCCTATGGTGATCCGGATTATTATGCGCTGCAGCTGTTCAATGGG
ATTTTTGGCGGTTTTTCACATTCGAAACTATTCATTAATGTCAGGGAGAAAGCCAGC
CTAGCGTATTATGCAGCTTCTAGGCTCGAAAGTCATAAAGGGCTATTGATGGTGATG
GCGGGCATCGAAAATGCGAACTACAAGCAAGCACTTGAAATCATTCATGCGCAAAT
GAATGAAATGAAACAAGGAAACTTTTCCGAGGCAGAACTGGAACAGACGAAGGCC
GTCGTAAGGAATCAGCTTCTGGAAACGATCGACGTTAGCAGGGGCCTCGTTGAAATT
TTATACCATAATGTAATTTCCGGGCAGAATATTTCACTGGACGATTGGTTTGCTGAA
ACGGAACGGACGACGAAGGAAGAAATTGTTGCCGTTGGTCAGAAGATTCAGCTTGA
TACAATTTATTTC**CTAACAGAAGCGGGGGTGCAAGAGTAA**

Reverse Complement Primer

The aLICator Cloning and Expression system was utilized to cloning SP1, P5, and ZNP2 gene targets. Primers with amino and carboxyl terminal histidine tags are displayed in Table 10. Primer combinations to generate no histidine tag, C-terminal histidine tag, and N-terminal histidine tag are displayed in Table 11.

Primer	Primer Sequence
SP1 pLATE 11F	AGAAGGAGATATAACTATGACAAGGGAAAAAAG ATTAATACC
SP1 pLATE 11R	GGAGATGGGAAGTCATTAATATTCCATGACCACTG ATTTACTCGG
SP1 pLATE31R	GTGGTGGTGATGGTGATGGCCATATTCCATGACCA CTGATTTACTCGG
SP1 pLATE52F	GGTTGGGAATTGCAAACAAGGGAAAAAAGATTAA TACC
P5 pLATE11F	AGAAGGAGATATAACTATGCACAAAAAGGATTGT TCCAAATCGG
P5 pLATE 11R	GGAGATGGGAAGTCATTATTCAGGCAGCCCGACA GCTTTAAACGC
P5 pLATE 31R	GTGGTGGTGATGGTGATGGCCTTCAGGCAGCCCG ACAGCTTTAAACGC
P5 pLATE 52 F	GGTTGGGAATTGCAACACAAAAAGGATTGTTCCA AATCGG
ZnP2 pLATE 11F	AGAAGGAGATATAACTATGTCTATCGCTTCCGATA CAATAATGG
ZnP2 pLATE 11R	GGAGATGGGAAGTCATTACTCTTGCACCCCCGCTT CTGTTAGG
ZnP2 pLATE 31R	GTGGTGGTGATGGTGATGGCCCTCTTGCACCCCCG CTTCTGTTAGG
ZnP2 pLATE 52F	GGTTGGGAATTGCAATCTATCGCTTCCGATACAAT AATGG

Table 10: Primers used for aLICator Cloning System. The aLICator cloning system was utilized to design SP1, P5, and ZnP2 primers to generate N-terminal and C-terminal histidine tags.

Protein Type	Primer Combination
No Histidine Tag Protein	11 Forward Primer + 11 Reverse Primer
C-Terminus Histidine Tag Protein	11 Forward Primer + 31 Reverse Primer
N-Terminus Histidine Tag Protein	52 Forward Primer + 11 Reverse Primer

Table 11: Primer Combinations for aLICator Cloning System. Primer combinations used to generate a C-terminal or N-terminal histidine tag.

Utilizing the primers designed for the aLICator cloning system, SP1, P5, and ZnP2 PCR amplicons were generated. Specific 14-21 nucleotide single stranded 5’ and 3’ overhangs were

generated using T4 DNA polymerase. The reaction mixture was then incubated with the corresponding vector and ligated. Ligation reactions were transformed XL1-Blue competent cells using the Zymo and TransformAid Bacterial Transformation Kit. Transformations were plated on LB-ampicillin plates and incubated at 37 °C overnight. Colonies were observed the following day (Table 12). Due to the quantity of colonies present, 5 colonies of each reaction were inoculated into 5 mL of LB+ ampicillin broth and incubated at 37 °C overnight. Plasmid DNA isolated and sequenced at Idaho State University Molecular Research CORE Facility using LIC Forward and Reverse primers.

Plasmid + Gene Insert	Zymo Kit	TransformAid Kit
SP1 + no histidine tag	Hundreds of white colonies	22 white colonies
SP1 + C- terminal histidine tag	Hundreds of white colonies	Hundreds of white colonies
SP1+ N-terminal histidine tag	Hundreds of white colonies	38 white colonies
P5 + no histidine tag	Hundreds of white colonies	8 white colonies
P5 + C-terminal histidine tag	Hundreds of white colonies	8 white colonies
P5 + N-terminal histidine tag	Hundreds of white colonies	5 white colonies
ZnP2 + no histidine tag	Hundreds of white colonies	49 white colonies
ZnP2 + C-terminal histidine tag	Hundreds of white colonies	15 white colonies
ZnP2 + N-terminal histidine tag	Hundreds of white colonies	25 white colonies

Table 12: OmpA Transformations Utilizing Zymo Kit and TransformAid Kit with XL1-Blue Competent Cells. Using the aLICator Cloning System SP1, P5, and ZnP2 PCR amplicons were cloned into pLATE vectors and transformed into XL1-Blue competent cells using two different chemical transformation kits.

The aLICator Cloning system used two different hosts cells, one for isolating plasmid DNA for sequencing and a second for expression. Once the orientation of insert was confirmed, the plasmids were transformed into host strain BL21 (DE3) using both the Zymo Kit and TransformAid kit (Table 13). Five colonies from each reaction were inoculated into 5 mL LB-

ampicillin broth. Culture incubated overnight at 37 °C. Once turbid, standard glycerol stocks were prepared as previously described.

Plasmid + Gene Insert	Zymo Kit	TransformAid Kit
SP1 + no histidine tag	4 white colonies	10 white colonies
SP1 + C- terminal histidine tag	50 white colonies	6 white colonies
SP1+ N-terminal histidine tag	50 white colonies	4 white colonies
P5 + no histidine tag	38 white colonies	2 white colonies
P5 + C-terminal histidine tag	27 white colonies	4 white colonies
P5 + N-terminal histidine tag	15 white colonies	N/A
ZnP2 + no histidine tag	5 white colonies	N/A
ZnP2 + C-terminal histidine tag	11 white colonies	12 white colonies
ZnP2 + N-terminal histidine tag	23 white colonies	25 white colonies

Table 13: OmpA Transformations Utilizing Zymo Kit and TransformAid kit with BL21 (DE3) Competent Cells. Isolated pLATE vector with ligated PCR amplicons were transformed into second competent host cell using two different chemical transformation kits. Samples with the comment "N/A" did not produce successful results following sequencing at MRCF. Transformed cells then used for preliminary expression experiments.

Preliminary expression experiments were conducted with only the SP1 cultures. Two cultures of SP1 no histidine tag, C-terminal histidine tag, and N-terminal histidine tag were inoculated into 5 mL LB-ampicillin broth. Once turbid, the cultures were induced with IPTG Supernatants were separated from the pellets and resuspended in 10 mM Tris with glass beads, placed in a bead beater, and the cell membrane disrupted as previously described. The samples were examined using PAGE and RSS. The molecular weight of SP1 is approximately 36 kDa and faint bands were present between 35-40 kDa (Figure 7).

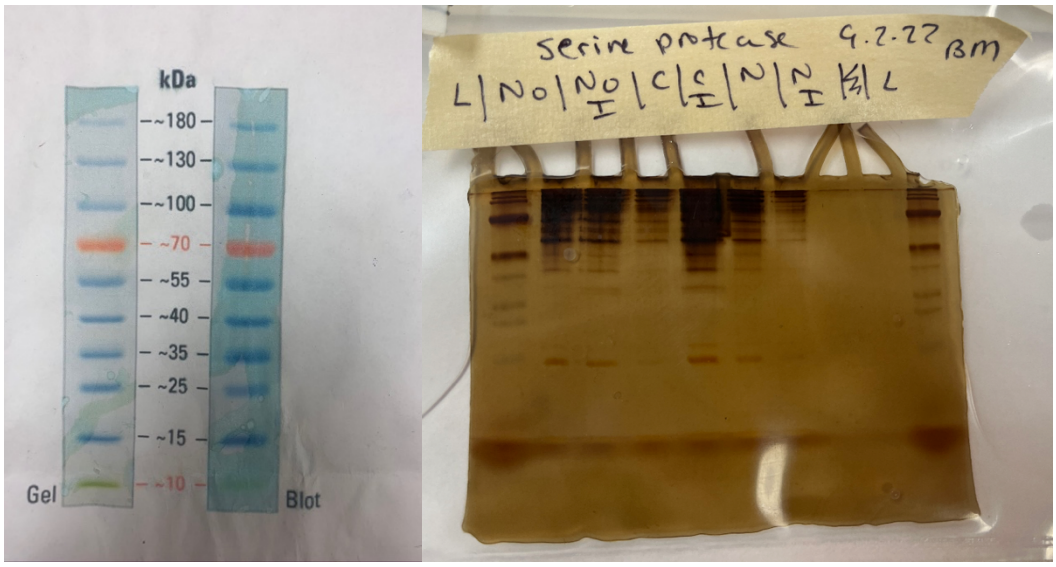

Figure 7: Sp1 Protein Expression from 5 mL Culture. Two cultures of each Sp1 No histidine tag, C-terminal histidine tag, and N-terminal histidine gat in host cells BL21(DE3) were inoculated in 5 mL LB-ampicillin broth incubated overnight at 37 C. Once cultures were turbid, one culture of each was induced with IPTG and incubated overnight at 37 C. Sample centrifuged, pellet resuspended in 10 mM Tris buffer, and the cell membranes disrupted using a Bead beater. From left to right: protein ladder, SP1 No histidine tag uninduced, SP1 No histidine tag induced, Sp1 C-terminal histidine tag uninduced, SP1 C-terminal histidine tag induced, SP1 N-terminal histidine tag uninduced, and SP1 N-terminal histidine tag induced. SP1 protein has a molecular weight of 36 kDa, however, bands within the target region were to faint.

Purification of the protein of interest was attempted using a rapid nickel affinity purification protocol (Figure 8).

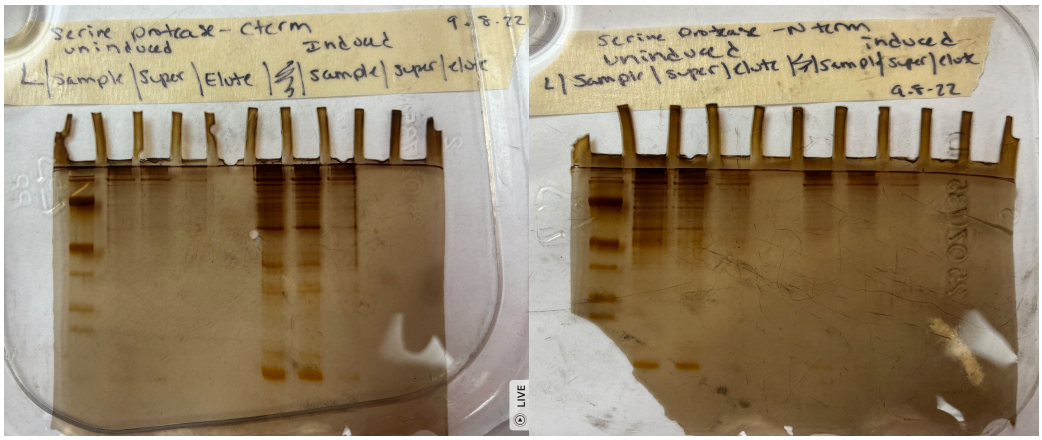

Figure 8: Rapid Nickel Resin Purification of SP1. In a 2 mL screw cap tube nickel resin and the uninduced and induced C-terminal or the N-terminal lysate added. Sample placed on a rotary shaker for 60 minutes, followed by 30 minutes on bench top to allows resin to settle to the bottom of the tube. Supernant collected and imidazole used to elute the proteins from the resin. The sample, supernatant, and eluate analyzed via PAGE followed by RSS. In the induced sample of C-terminal histidine tag, faint bands are present at 35-40 kDa.

Discussion

SOS isolates were struck on M9-Skim Milk and R2 + X-gal agar plates. A zone of clearing surrounding colonies on M9-Skim Milk agar indicates proteolysis of casein. Based on the results in Table 8, only one SOS isolate (SOS 1C-A3) exhibited a zone of clearing. R2 + X-gal agar plates were utilized to detect the presence of β-galactosidase. X-gal is a chromogenic substrate of β-galactosidase. When β-galactosidase is present, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl which dimerizes to form an insoluble blue pigment known as 5,5'-dibromo-4,4'-dichloro-indigo. Colonies appearing blue on R2 + X-gal agar plates, have β-galactosidase. The colonies on M9-Skim Milk with a zone of clearing and blue colonies from R2 + X-gal agar plates were sub-cultured to R2 media.

Growth on R2 media was documented (Table 9). Isolated colonies were inoculated into R2 broth for genomic DNA extraction. Once genomic DNA was extracted 16S rRNA analysis was performed on each of the isolates. The sequencing data was used to conduct a Basic Local Alignment Search Tool (BLAST) search to identify the most closely related organism, *Peribacillus frigoritolerans*. However, the genome was incomplete. The most closely related organism with a complete sequenced genome was *Peribacillus muralis*. Within the genome we identified 3 genes and target proteins SP1, P5, and ZnP2. Primers with amino and carboxyl terminal histidine tags were designed starting from the start codon to the stop codon of each gene target (Table 10; Table 11).

The aLICator Ligation Independent Cloning (LIC) and Expression system is designed for the expression of toxic proteins and ensures 95% cloning efficiencies. The system eliminates the use of restriction enzymes and ligation reactions. The three gene targets Sp1, P5, and ZnP2 no histidine tag, C-terminal histidine tag, and N-terminal histidine tag were cloned into the aLICator

system and transformed into E. coli XL1-Blue competent cells for sequencing the plasmid and then into E. coli BL21(DE3) for expression. Transformations were conducted using two transformation kits, the Zymo and TransformAid Bacterial Transformation kits, to assess the efficiency of each. For each of the three gene targets (no histidine tag, carboxyl terminal histidine tag, and amino terminal histidine tag), hundreds of white colonies were present following the initial transformation into XL1-Blue competent cells (Table 12). Plasmid DNA isolated and sent to the Idaho State University Molecular Research Core Facility (MRCF) and the insertion and orientation of the desired inserts were confirmed. Transformations were repeated with the expression vector BL21 (DE3; Table 13). Based on the transformations, both the Zymo and Bacterial TransformAid Kit performed successfully.

Of the three gene targets Sp1, P5, ZnP2 were successfully cloned and transformed into XL1-Blue and BL21 (DE3) cells. Preliminary expression experiments were conducted on SP1, however further testing is required to optimize expressions. Cultures containing the plasmid + SP1 insert were induced with 100 mM IPTG for a specified period of time. The cell membrane was then disrupted using a bead beater and the cellular contents were released into the extracellular environment. The supernatant was collected and run on PAGE gels. SP1 has a molecular weight of approximately 36 kDa, bands were present between 35-40 kDa (Figure 7). Purification of SP1 carboxyl and amino terminal histidine tag was attempted using a rapid nickel affinity purification protocol. Following purification, faint bands were present in the elute of the induced sampled.

Chapter III: Future Directions

The overreaching goal of the project was to identify proteases from *Peribacillus frigoritolerans*. The research resulted in three target genes (SP1, P5, and ZnP2) being amplified, cloned, and transformed into competent *E. coli* cells. Each gene was amplified with primers designed to add a C-terminal or N-terminal histidine tag. Utilizing the aLICator LIC Cloning system, the amplicons were cloned into pLATE vectors and transformed into XL1-Blue and BL21 (DE3) competent cells. Preliminary expression experiments were conducted however, further investigation is required to optimize protein expression. Once protein expression and purification are optimized the proteinases will be used in a series of experiments to analyze if they can break down keratin in chicken feathers.

Based on the results from the R2 + X-gal agar plates, β-galactosidases are present from the SOS isolates. Future work includes identifying the genes encoding the β-galactosidases, designing primers, amplification, cloning, and optimizing expression. Once protein expression and purification are optimized, the efficiency of lactose hydrolysis will be assessed.

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