Identification of secreted metalloprotease from the culture supernatant of *Mycobacterium bovis* BCG

By

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Abstract

Tuberculosis is considered one of the most prevalent diseases worldwide, claiming the lives of approximately 1.2 to 1.5 million people each year. It is caused by a member of a class of bacteria called *Mycobacterium* (*M. tuberculosis*), which includes saprophytic and pathogenic species. *M. bovis* causes similar disease in cattle. This latter species is used for the production of all currently available tuberculosis Bacillus Calmette-Guérin (BCG) vaccines. There has been progress made in the past 20 years in understanding the pathogenesis of Tuberculosis but much more research is needed to fully understand the mechanisms that mediate the survival of the pathogen in phagocytic cells. Metalloproteases are a subfamily of proteases that utilize metals, mostly zinc, for their catalytic activities. They are involved in virulence, cell wall processes and intermediary metabolism in bacteria. Metalloprotease activity in the culture supernatant of *M. tuberculosis* has been detected previously in other laboratories and we are able to detect this activity in the culture supernatant of *M. bovis* BCG. Bioinformatics analysis of the proteome of *M. tuberculosis* reveals a large number of putative proteases (66 in total) with 23 having the zinc-binding motif (PROSITE PDOC00129). The goal of this study was the direct identification of the protein responsible for the protease activity in the culture supernatant of *M. bovis* BCG. The protease in the supernatant was identified by zymography, with partial purification of the protease activity by ion exchange chromatography. A band on electrophoresis gels associated with the protease activity was then subjected to N-terminal sequencing of the purified protein, which identified hexapeptide sequence VTGGGA. Using a bioinformatics approach, the best matching protein with this sequence was identified as PstS, a phosphate binding protein normally considered a component of the phosphate transport system.

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Abs Absorbance
APS Ammonium persulfate
ATP Adenosine Triphosphate
BCG Bacillus Calmette-Guérin
Clp Caseinolytic
CR Complement receptors
DTT Dithiothreitol
EEA1 Early endosomal antigen 1
GSP General Secretion pathway
HIV Human immunodeficiency virus
HbN haemoglobins
h Hours
IM Inner membrane
KDa Kilo Dalton
L Liter
LAM Lipoarabinomannan
LC MS/MS Liquid chromatography–Mass Spectrometry
LTBI Latent Tuberculosis Infection
LAMP Lysosomal associated membrane protein
M Molar
µl Microliter
MDR Multi-drug resistant
mg Milligram
min Minutes
ml Milliliters
mM Millimolar
M. tuberculosis *Mycobacterium. tuberculosis*
MALDI MS Matrix Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometer
MW Molecular weight
MR Mannose receptors
OM Outer membrane
PI3P Phosphatidylinositol 3-phosphate
RNS Reactive nitrogen species
ROS Reactive oxygen species
RPM Rotations per minute
RT Room temperature
Sec Second
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STPK Serine/threonine-protein kinase
TCA Trichloroacetic acid
TEMED Tetramethylethylenediamine
Tris Tris(hydroxymethyl) aminomethane
v/v Volume per volume
w/v Weight per volume
WHO World Health Organization

1. Introduction

1.1 Tuberculosis

Mycobacterium tuberculosis is a slow growing, acid-fast bacterium possessing a complex cell envelope. It is the causative agent of the pulmonary infection tuberculosis (TB). Generally, *M. tuberculosis* infects the respiratory system but advanced disease can show other affected areas such as the skin, circulatory, lymphatic, central nervous and gastrointestinal systems (Plorde, 2004).

Tuberculosis is the second leading cause of death due to infectious diseases next to human immunodeficiency virus (HIV) with 1.2 to 1.5 million deaths annually. *M. tuberculosis* infections occur globally. The World Health Organization (WHO) estimates that one third of the world's population becomes infected with *M. tuberculosis* every year. As reported by WHO in 2014, there were about 9.6 million newly reported cases of TB and 1.5 million deaths due to TB; TB- linked HIV cases were reported to be about 1.2 million (WHO, 2015). TB coupled with HIV infection has become a leading cause of death for people suffering from HIV. HIV-positive patients are 20 to 30 times more prone to infection with *M. tuberculosis*. WHO reported an estimated 480 000 cases of multidrug-resistant TB (MDR-TB) worldwide in 2014, as well as, there were an estimated 190 000 deaths from multi-drug resistant tuberculosis, MDR-TB (WHO, 2015).

Tuberculosis is an airborne disease. The pathogen attacks the respiratory system and other organs including the kidneys, bones, brain and larynx. A droplet nucleus is the term used for the airborne particle that carries the bacteria in aerosols of exhaled or ejected respiratory exudate. When a person infected with pulmonary TB disease sneezes, coughs, sings or shouts,

infectious droplet nuclei are generated which remain in the air for hours. A healthy person gets infected when he or she inhales the infectious droplets via the nasal passages, mouth, upper respiratory tract or bronchi to reach the alveoli in the lungs. Initial exposure to the pathogen does not always result in infection and disease. Some individuals successfully overcome the pathogen without ever realizing they were infected. Others become infected, but the infection does not develop into clinical disease with the familiar symptoms of chronic cough and weight loss. This condition can be termed as latent TB infection. Currently, the point-of-care (POC) testing for TB is a skin test termed the tuberculin skin test. Patients with a latent TB infection are not contagious; nevertheless, they are at risk of developing clinical TB at some point. An estimated 5 to 10 percent of patients develop TB at some point of time in their lives if no preventative measures are undertaken (CDC, 2011).

Pulmonary TB exhibit symptoms that include cough coupled with cloudy, thick and, at times, bloody mucus from the lungs. Chills, fever, weakness, shortness of breath, chest pain and night sweats are also common symptoms. Other anatomical locations of TB infections that manifest symptoms including skin sores, abdominal pain, swollen lymph nodes, bone and back pain (Wejse et al., 2008). Tuberculosis can be diagnosed in various ways, including medical history, chest X-ray, different laboratory tests and physical examinations. Latent Tuberculosis infection (LTBI) can be identified through two laboratory tests, namely the Purified Protein Derivative (PPD) skin test and a TB blood test, also known as the interferon-gamma release assay (IGRA). In the PPD test five tuberculin units of PPD is injected into the forearm of the subject and, after 48 to 72 hours, the swelling at the site of the injection is measured (Ellner, 2011). The size of the skin reaction is used to determine potential TB infection (reaction of more than 12 mm in diameter is typically considered a positive reaction). In the TB blood test, the

measurement is done based on the reaction of the immune system towards bacteria causing TB or its components. The basis of this test is the activity of the effector T cells that react to stimulation caused by *M. tuberculosis* antigens (particularly ESAT-6 and CFP 10), causing the release of interferon gamma (IFN-γ) into the medium (Diel et al., 2011).

Treatment of the infection typically entails a six-to-nine month course of isoniazid. Other treatments may include daily rifampin plus pyrazinamide (taken for a period of 2 months) or daily rifampin alone for 4 months. These antimicrobials have the capability to kill the bacteria before they escalate to active TB (Lienhardt et al., 2010). At the onset of the last century, high hopes rested on the prevention of TB through the newly invented *M. bovis* bacilus Calmette-Guérin (BCG) vaccine. The original vaccine strain was developed by Albert Calmette and Camille Guérin at the Pasteur Institute (France) during the 1920s, following successive passaging of a clinical isolate of *M. bovis* in glycerinated medium containing ox bile (Fine, 1995). The original vaccine strain was cultured by several laboratories throughout the world and the laboratory passaging has produced at least 12 recognized variants today. Each of these became known as the strain developed in that particular geographic location; for example *M. bovis* BCG (Tokyo), *M. bovis* BCG (Pasteur), *M. bovis* BCG (Russia) (Zhang et al., 2013; Seki et al., 2009). These vaccines are generally meant for infants in various countries and have been successful, to a certain degree, in providing protection against intense forms of TB like miliary tuberculosis, and tuberculosis-induced meningitis. On the other hand, BCG offers only little protection against pulmonary TB. It thus does not play an effective part in the prevention of the pulmonary disease in adults. Why the effect of BCG vaccine on neonates starts to diminish in adults still remains unknown (Ottenhoff et al., 2012). Hence, there is a need to produce an

improved vaccine, which would contribute towards the prevention of pulmonary disease amongst adults.

1.2 *Mycobacterium tuberculosis*

In 1882, Robert Koch made the discovery of *Mycobacterium tuberculosis*. Due to the absence of an outer cell membrane and being stained by Ziehl-Neelsen stain, *Mycobacterium tuberculosis* is categorized as an acid fast Gram-positive bacterium (Plorde, 2004). As a large non-motile rod-shaped bacterium and as an obligate aerobe, *M. tuberculosis* requires oxygen in order to multiply. However, its duplication period is very slow when compared with other bacteria; it takes between 15 to 20 hours for the bacterium to replicate (Todar, 2008). Members of the *Mycobacteria* are categorized into two groups namely: slow growers and fast growers. The slow growers include *M .tuberculosis* and *M. bovis,* which cause bovine tuberculosis. The latter can transfer to humans if milk coming from an infected animal is consumed. *Mycobacterium leprae* is another member of the slow growing group and is the causative agent of leprosy. Non-pathogenic bacteria such as *M. smegmatis* are the fast growers (Forrellad, et al., 2013).

M. tuberculosis is a complex bacterium with a distinct cell envelope made of proteins, peptidoglycans, mycolic acids, lipids and carbohydrates (Wolfe, et al., 2010). The cell envelope of *M. tuberculosis* consists of a plasma membrane and a cell wall core containing peptidoglycans, arabinogalactan, and mycolic acid (Kaur et al., 2009). These layers are encircled by another layer of lipid-rich mycolates (Brennan & Nikaido, 1995). While this complex, hydrophobic, and highly impermeable barrier protects the pathogen from environmental hazards and host immune system attacks, there is a need for specialized secretion systems to allow the bacterium to release crucial virulence factors and enzymes to facilitate infection. *Mycobacteria*

possess the general secretion pathway (GSP or Sec-dependant), as all bacteria do. This process is facilitated by N-terminal signal sequences in the proteins that target them for secretion. *Mycobacteria* also utilize a Sec-independent secretion mechanism known as the twin-arginine transporter (Tat) pathway. Protein secretion in Gram-negative bacteria requires a transport process that will traverse the entire cell envelope made up of two membranes: the inner membrane (IM) and outer membrane (OM), with the periplasm positioned in between these two membranes. Basically, there are two ways of accomplishing secretion. The first is the one-step mechanism where the cell envelope is traversed in one event (type I secretion). The other is a two-step mechanism where distinctive machinery is employed to traverse the OM (Abdallah et al., 2007).

The pattern by which protein secretion takes place has been thoroughly studied in Gram-negative bacteria, apart from the Sec and Tat systems. An extensive study has identified the different specialized secretion systems (Type I to VI). In Type I secretion systems, proteins are simply transported across the cytoplasm to the outer milieu in a single step (Delepelaire, 2004). In Type II systems, Sec or Tat pathways transport proteins across the inner membrane, followed by processing through an outer membrane protein, such as secretin, that translocates proteins from the periplasm to the outer milieu (Cianciotto, 2005; Desvaux et al., 2004; Filloux, 2004). The Type III system, also known as the injectosome, allows bacteria to inject their virulence factors directly from the cytoplasm of the bacterium into the cytoplasm of the host cell (Mota & Cornelis, 2005). The Type IV system is best characterized as a Nano machine, similar to a conjugation system that transports proteins or DNA across the cell envelope of bacteria (Fronzes et al., 2009; Christie et al., 2005; Schröder & Lanka, 2005). The Type V secretion system simply uses a two-step mechanism. Proteins are first translocated across the inner membrane (IM) and

subsequently transported across the outer membrane (OM) through a transmembrane protein complex with a β-barrel configuration (Desvaux et al., 2004; Thanassi and Hultgren, 2000; Henderson et al., 1998). In Type VI secretion systems, proteins are injected directly into the host cell cytosol through the structure of phage-tail-spike-like injectosome, similar to the Type III and Type IV machineries (Filloux et al., 2008; Tseng et al., 2009). Some of the secretion systems in Gram-positive bacteria are simpler than those found in Gram-negative bacteria, due to the absence of a second membrane. In other words, the Gram-positive bacteria secretory proteins just need to be delivered to the extracellular milieu through passage across the cytoplasmic membrane and peptidoglycan layer. Despite the fact that most of the Gram-negative secretory systems are not present in the *M. tuberculosis* genome, their possible functional equivalent, known as ESX-1, is a recent discovery (Feltcher et al., 2010). In recent years, experimental evidence has shown that ESX-1 can be utilized as a replacement for specialized protein secretion systems in *M. tuberculosis* (Abdallah et al., 2007). The original idea of the existence of a specialized secretion system in *M. tuberculosis* was the result of studies that singled out the secreted proteins which have no apparent Sec signal sequences (Converse & Cox, 2005). There are two secreted proteins, without sec signal sequences, in *M. tuberculosis* with unknown functions yet their cell targets are vital and crucial for *M. tuberculosis* virulence. The two secreted proteins are ESAT-6 (Early secreted antigen target 6 kDa) and CFP-10 (Culture filtrate protein, 10 kDa). These findings are indications of the very complex nature of the ESX-1 system. Recent findings suggest that multiple protein complexes are required in order for the ESX secretion mechanism to function properly (Champion & Cox, 2007). During *M. tuberculosis* infection, the ESX-1 secretion is essential for a proper macrophage cytokine response and for balancing early contact with the host cell. It is also responsible for regulating the immune

response of the host cell (Stanley et al., 2003). There seems to be some similarities between the ESX-1 system and the Type IV secretion systems (Champion & Cox 2007).

1.3 Phagocytosis and Intracellular Pathogenesis

The process of phagocytosis of microorganisms by macrophages is an important component of innate immune response. Shortly following their formation, phagosomes are processed by a series of interactions with endosomes leading to the maturation of the phagosome with eventual fusion with lysosomes to form phagolysosomes. The process is called phagolysosome biogenesis whereby phagolysosomes obtain degradative and microbicidal characteristics (Vergne et al., 2004). It is an efficient microbe neutralizing process for the most part, but has proven to be ineffective with pathogens such as *M. tuberculosis* and other pathogens. *M. tuberculosis* can survive this defensive mechanism and block the biogenesis of the phagolysosome (Vergne et al., 2004). Furthermore, obstructing phagolysosome biogenesis might help *M. tuberculosis* propagation within infected macrophages, using the macrophages as vehicles to move within the host, shielded from the immune system (Deretic et al., 2006; Vergne et al., 2004).

Meena and Rajni (2010) propose that *M. tuberculosis* has evolved effective survival various mechanisms. These include blocking phagosome-lysosome fusion during phagocytosis, inhibition of phagosome acidification, avoiding the delivery of phagosome wall coat proteins to lysosomes, resisting reactive oxygen and nitrogen radicals, and expression of glycine-rich proteins (Figure1).

Russell (2001) and Pieters (2001) stated that mycobacterial entry into phagocytic cells can be attributed to binding to different phagocytic receptors, thereby resulting in uptake and delivery of

the microbe into host phagosomes. These receptors may include complement receptors (CR) (Cywes et al., 1997), mannose receptors (MR), Fc receptors and scavenger receptors (Ernst, 1998). Once inside phagosomes, *M. tuberculosis* eliminates a set of host molecules and produces a number of virulence factors, thus inhibiting the maturation of phagosomes into phagolysosomes. The ultimate result of the maturation of the phagosome is to subject the internalized microbe to an acidic environment, whereby digestive enzymes can be activated at low pH to help degrade microbe components and activate the respiratory burst. (Kinchen & Ravichandran, 2008). The latter is the process by which reactive oxygen (ROS) and nitrogen species (RNS) form within the phagolysosome. Attack by the digestive enzymes and the activity of ROS and RNS ultimately results in neutralizing and degrading the microbe. Starting from internalization of the pathogen to degradation in the phagolysosome, there are three steps of phagosomal maturation that take place in macrophages, resulting in the formation of early, intermediate, and late phagosomes. In early phagosomes, a near neutral pH of 6.3 is produced due to activity of vacuolar H^+-ATPases pumping protons into the phagosome (Welin, 2011; Russell, 2007). There are two types of proteins that have crucial roles in phagosomal maturation processing which are EEA1 (early endosomal antigen 1) and Rho-GTPase Rab5 (Seto et al., 2011). In the intermediate phagosome, the EEA1 protein is absent from the phagosomal membrane whereas Rab5 is involved in this step. In late phagosomes, LAMP (lysosomal associated membrane protein) and Rab7 from TGN (the trans Golgi network) are always found associated with the phagolysosmal membrane. Upon further acidification of the phagolysosome, cathepsin D is activated by cleavage, and this is using as an identifying marker for the stage of phagosome maturation (Flannagan et al., 2009). The last stage of the process involves a further acidification of the phagosome to reach a pH of 4.5-5 (Rohde et al., 2007). The phagosomal

maturation process takes approximately 90 minutes to complete from the initial step of pathogen engulfment (Rohde et al., 2007).

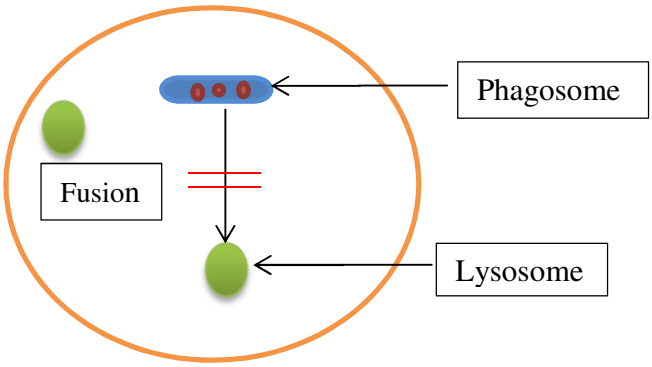
Phagosome maturation and fusion events, as in other intracellular vesicles, depend on vesicle mobility within the cell. This mobility is facilitated by the cytoskeleton and a variety of cytoskeleton- and vesicle-associated proteins. Two such proteins are the Rab7A and the Rab-interacting lysosomal protein (RILP) (Harrison et al., 2003). While many of the phagosomal maturation steps are Ca^{2+} dependant, Zimmerli et al., 1996 observed that Ca^{2+} was not required for phagosome maturation. Moreover, Malik et al., (2003) concluded that the phosphatidylinositol-3 kinase (PI3K)-independent route was used to deliver LAMP-1 to the phagosome while Ca^{2+} plays an exclusive role in the PI3K-dependent pathway. The formation of the phagolysosome is the last step of phagosome maturation. The phagolysosome is an extraordinarily extraordinary pathogen killer with digestive enzymes and other elements that diminishes the effect of the pathogen.

M. tuberculosis uses multiple mechanisms to evade killing and generate a suitable environment for replication and inhibition of phagosomal maturation. Several products of this pathogen have been shown to play a role in blocking phagosomal maturation, including LAM, SapM, and protein kinase G (Mueller & Pieters, 2006). LAM (lipoarabinomannan) is a major cell wall component and has an important role in pathogen survival following phagocytosis (Guerardel et al., 2003). It blocks the phagosome maturation through two pathways, which include phsophatidyl-insotiol-3 phosphate and the phosphatidylinositol-3 kinase (Fratti et al., 2003). As mentioned earlier, virulence factors of the pathogen can prevent EEA1 from associating with the phagosomal membrane, a required step for phagosome maturation (Rajni et al., 2011). LAM can inhibit the elevation of intracellular Ca^{2+} which, in turn, activates the

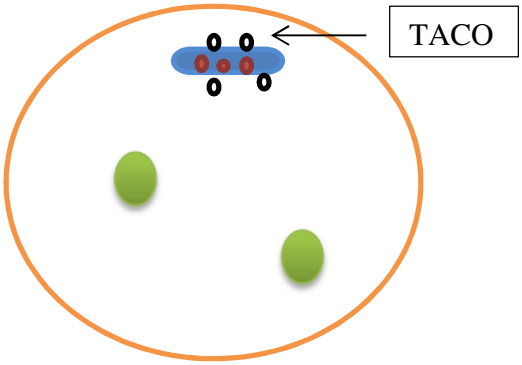
Ca^{2+}/calmodulin- dependent PI3K hVPS34 (Vergne et al., 2003), the activity of which can regulate the association of several vesicle fusion proteins with the phagosomal membrane (Rajni et al., 2011). Another *M. tuberculosis* product that affects phagosome maturation is SapM, which is a secreted acid phosphatase. This secreted protein has been shown to dephosphorylate PI3P (Saleh and Belisle, 2000). Dephosphorylation of PI3P by SapM and inhibition of PI3P hVPS34 by ManLAM are essential for inhibition of EEA1 association with the phagosomal membrane. Protein kinase G (PknG) is another secreted virulence factor that plays an important role in the survival of *M. tuberculosis* in the presence of macrophages (Walburger et al., 2004), although the actual host target of this kinase is unknown. *Mycobacteria* can also block phagosome fusions with lysosomes through the tryptophan/aspartate containing coat protein, also known as TACO (Gatfield et al., 2005). Mueller and Pieters (2006) state that this protein possibly forms a shield around phagosomes blocking fusion with lysosomes (Fig.1).

Phagosomes are designed to exclude iron as much as possible, thus limiting this crucial nutrient from the internalized pathogen (Rhoades & Ullrich, 2000). Acidic pH inside the phagosome is essential for acquiring iron from transferrin (Ismail, et al. 2002). Production of ROS and RNS inside activated macrophages constitutes another important antimicrobial mechanism. During phagocytosis, phagocyte oxidase assembles within the phagolysosomal membrane into an enzymatically active complex that breaks down molecular oxygen into superoxide anions (O^{2-}) (Ehrt and Schnappinger, 2009). Superoxide anions dismutate into hydrogen peroxide (H_2O_2) and produce toxic hydroxyl radicals (•OH). Inducible nitric oxide synthase (iNOS) is induced by IFNγ to create nitrate (NO_{3-}) and nitrite (NO_{2-}) through nitric oxide (•NO). These compounds can be converted into nitrous acid and nitrogen dioxide ($•NO_2$) under acidic conditions (Nathan & Shiloh, 2000). As Ehrt and Schnappinger (2009) state, the

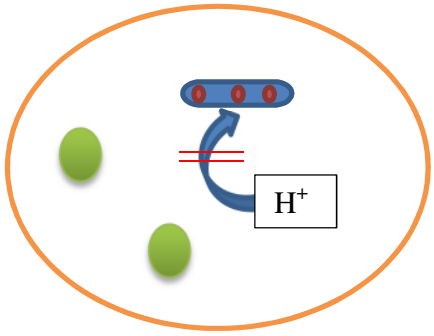
reaction between nitric oxide (ON) and superoxide produced by macrophages generates toxic peroxynitrite ($ONOO^-$).Various processes such as apoptosis, cytokine response, neurotransmission, iron metabolism and cell proliferation involve the production of ROS and RNS. In addition to ROS and RNS, there are antimicrobial proteins inside the phagosome which are able to kill the pathogen. These proteins (lysozymes, lipases, proteases, and hydrolases) have the ability to make the components of pathogen's interior critical to its viability accessible by degrading the pathogen's envelope (Welin, 2011).



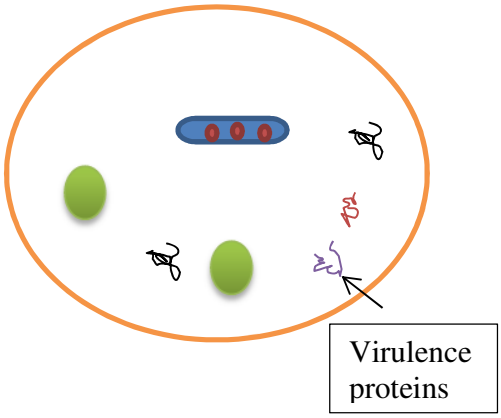
Inhibition of phagosome–lysosome fusion



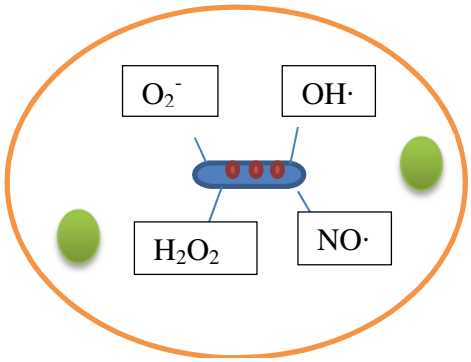
Tryptophan aspartate which is a coat protein on phagosome



Inhibition of acidification of phagosome



Expression of virulence proteins



Protection form reactive oxidative radicals

Figure 1: Postulated mechanisms employed by *M. tuberculosis* to survive inside macrophages (Meena and Rajni, 2010).

There are additional mechanisms by which *M. tuberculosis* escapes the protective measures of its host to promote its survival within the host. The generation of biological molecules that detoxify ROS and RNS, enable protein repair, and protein degradation are some of these mechanisms. For example, the effect of methionine oxidation can be repaired by Methionine sulfoxide reductase (Msr). There are two cell envelope enzymes produced by *M. tuberculosis* that are required for pathogen growth within the host and causing disease. These enzymes are superoxide dismutase and catalase-peroxidase. Superoxide dismutase transforms cell damaging superoxide anions into hydrogen peroxide. Moreover, catalase-peroxidase decomposes the hydrogen peroxide into water and oxygen to promote pathogen growth. *M. tuberculosis* also produces mycothiol that protects it from oxidative stress (Bhaskar et al., 2014). Additionally, strong activity of nitric oxide dioxygenase (NOD) of *M. tuberculosis*, by the truncated hemoglobin (HbN), has been shown to efficiently deactivate RNS (Lama et al., 2009). Ehrt and Schnappinger (2009) have indicated that *M. tuberculosis* coenzyme F-420 is used to protect against nitrosative damage as well. Also the ROS and RNS detoxifying enzymes NADPH-dependent peroxidase and peroxynitrite reductase (coded by the *M. tuberculosis* genome) have been identified as playing potential protective roles during infection (Ehrt & Schnappinger, 2009).

1.4 Proteases

Proteases, enzymes that catalyze peptide bond cleavage in proteins, are categorized on the basis of their catalytic site as threonine peptidases, metallo-peptidases, cysteine peptidases, aspartic peptidases, glutamic acid peptidases, serine peptidases, mixed catalytic-type peptidases, and unknown catalytic-type peptidases (reviewed by Polgar, 2005). Plants and animals express proteases; however, microorganisms are an abundant source of these enzymes and play various

roles in normal microbial physiology, metabolism, and pathogenesis (Rao et al., 1998). Some of the microbial proteases represent crucial virulence elements for pathogens. Proteases regulate specific cellular proteins in the pathogen and host and are involved in replication, metabolism, transcription, and cellular homeostasis (Butler et al., 2006). They also contribute to the virulence of pathogens by degradation of host proteins such as extracellular matrix proteins (Hu Q et al., 2010). In addition, there are several additional roles that pathogen proteases play in the host cell. For example, the protease from *Porphyromonas gingivalis* provides a source of amino acids for the pathogen by hydrolyzing host proteins (Kuramitsu, 1998). They can also interfere with normal host events, like signal transduction, cell cycle, cell differentiation, apoptosis and motility (Elkind et al., 2010). *Neisseria gonorrhoeae* secrets IgA1 protease that cleaves human lysosome-associated membrane protein (h-lamp-1) resulting in the destruction of the phagosomal membrane (Hauck &Meyer, 1997).

***M. tuberculosis* Proteases:**

The genome of *M. tuberculosis* H37Rv encodes about 100 proteases. Some are probable secreted proteins (Cole et al., 1998) and 38 are conserved amongst *M. bovis*, *M. leprae*, *M. tuberculosis* and *M. avium paratuberculosis.* Little is known about the biology of these aforementioned enzymes in these pathogens (Ribeiro-Guimarães and Pessolani, 2007). Some classes of *M. tuberculosis* proteases include:

I) Serine Proteases:

This type of enzyme cleaves peptide bonds in proteins; serine plays the role of a nucleophile at the active site. They are known to coordinate different physiological functions in eukaryotes and prokaryotes, which include blood coagulation, digestion,

reproduction and immune response (Polgar, 2005). There exists a family of five subtilisin-like serine proteases that include mycosins 1 to 5, which have a high similarity to those encoded by *M. tuberculosis* H37Rv (Brown et al., 2000). An important family member, MycP1 can be detached from the membrane fraction or cell wall and is not expressed in the attenuated *M. bovis* strain BCG, although it has the MycP1 gene (Dave et al., 2002). The ESX-1 secretion system in *M. tuberculosis* requires MycP1, a protein that is essential for the replication of the pathogen inside the macrophage and for virulence in mice. Additionally, ESX-1 protein export is regulated by MycP1 by splitting EspB, one of the substrates of the ESX-1 secretion system. EspB can boost secretion of ESX-1 substrates and its proteolysis by MycP1 plays a role in suppressing and/or regulating secretion. Thus, MycP1 protease activity negatively controls secretion through EspB, which is needed for the functioning of the ESX-1 system (Ohol et al., 2010).

II) ATP-depended proteases:

The majority of bacteria have this type of protease which is an energy-dependent (De Mot et al., 1999). The most common energy-dependent proteases are called Caseinolytic (Clp) proteases (Personne et al., 2013). *M. tuberculosis* is known to possess two ClpP proteases homologs, ClpP2 and ClpP1 within a single operon which are also associated with ATPase chaperones ClpX and ClpC (Raju et al., 2012). There are several roles of ClpP2 and ClpP1 *in vivo*. It is crucial for growth and the other role was observed through the knockdown strain of ClpP1P2 that was done by Carroll et al., (2011), which diminishes growth and absence of propagation after macrophage

infection. Furthermore, the degradation of irregular proteins generated in the presence of certain antibiotics requires ClpP1 and ClpP2 activity (Raju et al., 2012).Clp proteases degrade the proteins by assembling ClpP and Clp ATPases into a complex requiring ATP. After that, the unfolded proteins are recognized and bound by Clp ATPases. Next, the unfolded proteins are degraded after binding with ClpP. Finally, degraded proteins are liberated in the cytoplasm (Roberts et al., 2013). . As a matter of fact, during *M. tuberculosis* growth within the macrophage, clgR, which is a transcriptional activator of the clpP1P2 operon, is activated (Estorninho et al., 2010). A critical aspect of *M. tuberculosis* physiology is targeted by small molecule modulators of ClpP1P2 activity (Raju et al., 2012).

III) Zinc- dependent Metalloproteases

Metalloproteases commonly use zinc as metal to support catalysis. They are further required in intermediary metabolism, virulence and cell wall processes (Forrellad et al., 2013). Relying on the peptide sequence HExxH which is an active site motif for all zinc-metalloproteases, there are three putative Zn-dependent metalloproteases encoded by the *M. tuberculosis* genome which are Rv2869c (Rip), Rv0198c (Zmp1), and Rv1977 (Zhao & Xie, 2011). Rv1977 is a Zn-dependent enzyme that is a putative aminopeptidase with a chaperone function. However, this protease is not likely involved in virulence due to its absence in *M. bovis* (Forrellad et al., 2013).

Zmp1 (Rv0198c) play a critical role in *M. tuberculosis* pathogenesis, since it inhibits phagosome maturation (Ferraris et al., 2011). Furthermore, Rv0198c may play a role

in infection by blocking the activation of the inflammasome (Muttucumaru et al., 2011).

M. tuberculosis is capable of checking phagosome maturation by arresting the inflammasome (Master et al., 2008). Guarda and So (2010) state that the inflammasome is a mixture of sensor cytoplasmic proteins that are activated when a pathogen is detected, leading to activation of pro-caspase-1. The activation of Pro-IL-1β into the inflammatory cytokine IL-1β results from the proteolytic effect of caspase-1. The phagolysosome fusion and early reaction of the inflammatory response are triggered in an autocrine and paracrine manner when IL-1β is expressed (Master et al., 2008). The work of Master et al. (2008) showed that Zmp1 arrests phagosome maturation through inhibition of the inflammasome which blocks the cleavage of pro-IL-1β into active IL-1β. Therefore, *M. tuberculosis* secrets Zmp1, where it inhibits the inflammasome and phagosome maturation (Lazarevic & Martinon, 2008; Master et al., 2008).

The virulence of *M. tuberculosis* was shown to be enhanced by a RIP metalloprotease (Rv2869, or Rip1) (Makinoshima & Glickman, 2005). A method of transmembrane signal transduction, regulated intramembrane proteolysis (RIP) results in the cleavage of specific intramembrane substrates and release of one or more cleavage products (Sklar et al., 2010). The role of zinc-metalloproteases in microorganism pathogenicity is pointed out in different studies; one such study was conducted by Denkin and Nelson (2004). The study revealed that an extracellular zinc metalloprotease, EmpA, is considered a virulence factor that plays a role in the pathogenesis of the fish pathogen *Vibrio* anguillarum; EmpA was also identified in another study as a significant virulence factor that would help the pathogen invade host defenses (Denkin & Nelson, 2004).

Moreover, anthrax lethal factor, a zinc-metalloprotease and an important virulence factor of *B. anthracis*, was shown to invade host cells and cleave mitogen activated protein kinase (MAPK) kinases required for growth (Duesbery et al., 1998).

1.5 *Mycobacterium bovis BCG*

Mycobacterium bovis (*M. bovis*) is described as the etiological agent of bovine tuberculosis. It has a high degree of genetic similarity with *M. tuberculosis* responsible for human tuberculosis. In addition, the transmission of the bacteria from cattle to humans causes disease (Garnier et al., 2003). *M. bovis* can attack humans primarily through the ingestion of unpasteurized dairy products. Transmission through aerosol inhalation that follows close contact with infectious cattle and infection from person to person is rare. Grange (2001) suggests that uncooked or raw meat could also be a potential source of the organism.

In France, between 1908 and 1921, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine was invented by Albert Calmette and Camille Guérin. It is a live refined vaccine obtained from *M. bovis*. Annually, there are more than 120 million doses provided around the world, which make this vaccine the most widely used. The effect of the BCG vaccine is reduced after 13 years and 230 culture cycles; however, it was able to prevent infection of calves and guinea-pigs with the virulent strain of *M. tuberculosis* (Ritz et al., 2008). BCG provides effective immunization in children, with 80 percent protection against serious forms of tuberculosis such as miliary disease and tuberculous meningitis (Trunz et al., 2006). Additionally, BCG exerts significant favorable effects on infant viability (Roth et al., 2006). This research encourages adoption of *M. bovis* BCG as a model for vaccination because of the close homology between virulent *M. tuberculosis* and the attenuated *M. bovis* BCG, which stands at 99.95% identity at the

nucleotide level (Garnier et al., 2003), and shows that the latter is capable of secreting a number of virulence factors also secreted by *M. tuberculosis*.

1.6 Hypothesis

Metalloproteases secreted by *M. bovis* BCG are likely the same as in *M. tuberculosis* and likely play a role in blocking phagosome-lysosome fusion during phagocytosis in macrophages, thereby allowing the pathogen to survive the process. The model shown in Figure 2 proposes a mechanism by which a secreted protease may inhibit the fusion of phagosome with lysosome.

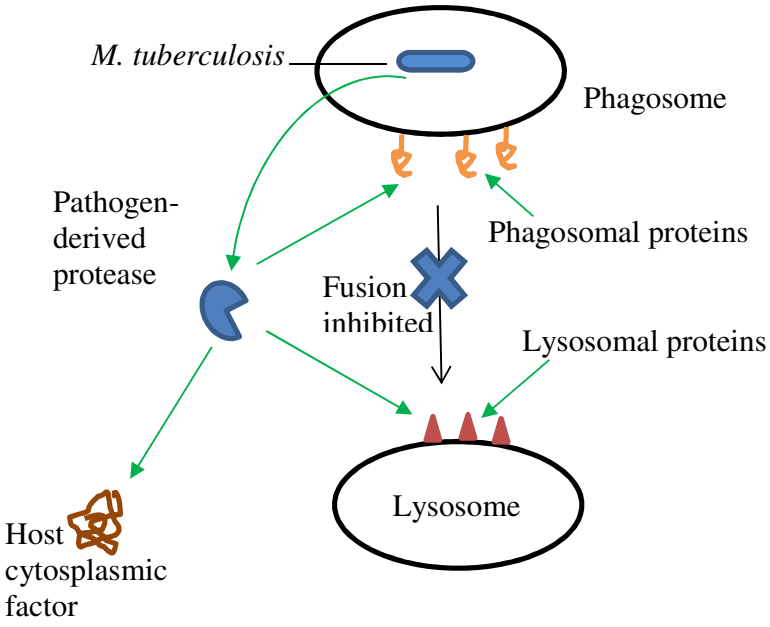


Figure 2: A hypothetical model describes the process by which a secreted protease of *M. tuberculosis* may access macrophage factors and inhibit the fusion of phagosome-lysosme. Some of these factors might be LAMP, Rap5, TACO, or another host factor not identified as of yet.

1.7 Rationale

The pathogenesis of the *M. tuberculosis* is multi-faceted and involves both pathogen and host factor interactions. Several pathogen-derived factors may contribute to the survival of this pathogen. In addition to the factors already described in the literature other factors may be involved in this process. A possible in this process exists for metalloproteases as some members of this group of enzymes have been implicated in a number of intracellular pathogens. In this study the TB vaccine bacterium *M. bovis* BCG was used to investigate the secretion of such proteases. *M. bovis* BCG has been utilized as a model organism to study the virulence of *M. tuberculosis* in human and animal cells as well as in various animal models. This bacterium is less virulent than the human pathogen but may still cause disease in a small fraction of those who receive the vaccine and particularly those with a compromised immune system. It is capable of secreting a number of virulence factors during infection and interacts with macrophages in a similar manner to *M. tuberculosis*.

1.8 Objectives

The main aim of this study is to confirm that *M. bovis* BCG secretes one or more metalloproteases and to identify proteases present in *M. Bovis* culture supernatants. This will be accomplished by:

- *In silico* analysis of the *M. tuberculosis* genome in order to identify potential secreted proteases.
- Isolation of candidate proteins associated with these protease activities and identification by mass-spectrometry.

2. Materials & Methods

2.1 Media

Sauton's media

In 500 ml of distilled water, 0.5 of each magnesium sulfate (Fisher Scientific, New Jersey, USA) and potassium phosphate (Fisher Scientific, New Jersey, USA) , 2. g citric Acid (Sigma Aldrich Co., ST. Louis ,MO, USA) and 0.05 g of ferric ammonium citrate (Fisher Scientific, New Jersey, USA) were dissolved. 4.0 g of ammonium chloride (Sigma Chemical Co., ST. Louis, USA) or L-asparagine (Sigma Aldrich Co., ST. Louis, USA) were added in addition to 60 ml of glycerol (Fisher Scientific, New Jersey, USA) and the volume was brought up to 1 L with distilled water and autoclaved.

Middlebrook 7H11 Agar Plates

In 450 ml distilled water, 10.15 g of Middlebrook 7H11 agar base (Sigma Aldrich Co., ST. Louis, USA) was dissolved utilizing heat and 2.5 ml glycerol were added before autoclaving. Once cooled, the agar was supplemented with one vial of OADC and 100 mm plates were poured in approximate 15 ml volumes.

2.2 Culturing Bacterial Strain

A) Mycobacterium bovis BCG

M. bovis BCG (Frappier) was cultured in Sauton's media at 37°C in a rotary shaker (Innova 4000 incubator shaker) from NEW BRUNSWICK SCIENTIFIC (120 rpm) for three weeks or on Middlebrook 7H11 agar plates also incubated at 37°C.

2.3 Measurement of protein concentration

The Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) was used as directed to quantify protein samples. This assay is based on bicinchoninic acid (BCA) and the reduction of Cu2+ to Cu1+ by means of the biuret reaction for colorimetric detection and quantification. Absorbance of samples prepared in triplicate were read in (FLUOstar OPTIMA) from BMG LABTECH (Offenburg, Germany) at 562 nm and compared to a set of standards also prepared in triplicate.

2.4 SDS PAGE

A) One dimensional SDS polyacrylamide gel

The one dimensional SDS polyacrylamide gels were made of a 12% separating gel [3.4 ml H_2O, 2.5 ml 1.5M Tris-HCl (Fisher Scientific, New Jersey, USA) pH 8.8, 50 µl 20% SDS(Bio Basic Canada, Ontario, Canada), 4 ml 30% acrylamide/0.8% bis-acrylamide (w/v) (Bio Rad, Hercules, California, USA), 60 µl 10% (w/v) ammonium persulfate (APS) (Fisher Scientific, USA), 7µl tetramethylethylenediamine (TEMED) (G.E. Health Care Quebec, Canada) and a 4% stacking gel [3.1 ml H_2O, 1.25 ml 0.5M Tris-HCl pH 6.8, 25µl 20% SDS, 0.65 ml 30% acrylamide/0.8% bis-acrylamide (w/v), 31 µl 10% (w/v) APS, 5 µl TEMED]. Protein samples (5-40µg) were combined with 2X sample buffer [38% glycerol, 3.8% SDS, 0.1M Tris-HCl pH 6.8, 0.02% bromophenol blue (Fisher Scientific ,New Jersey, USA)] to a final concentration of 1X and boiled for 5 min in a water bath. For non-reducing conditions, DTT was omitted and samples were not boiled. Protein in sample buffer was loaded into the wells and gels were run at a constant voltage of 150V for 100-120 min in a Bio-Rad (Mini-PROTEAN Tetra System) with 1X SDS running buffer [1/10 dilution of 30 g Tris-HCl, 144 g glycine, 10 g SDS in 1 L dH20;

pH 8.3]. The Thermo Scientific Prestained Protein Molecular Weight Marker was used as a protein standard for proteins ranging in size from 20 – 120 kDa (Laemmli, 1970).

B) Two dimensional SDS polyacrylamide gel

1) First Dimension

The first dimension is a native polyacrylamide gel. A 10% polyacrylamide was used for first dimension. Using the same protocol for one dimensional SDS polyacrylamide gel except SDS was omitted. The protein was mixed with 5X native sample buffer [15.5 ml of 1M Tris-HCl, pH 6.8, and 2.5 ml of 1% solution of bromophenol blue, 7.0 ml of distilled water and 25 ml of glycerol. The native running buffer contains [3.0 g of Tis base and the 14.4 g of glycine dissolved in dH2O] and adjust the volume to 1 L .The final pH is 8.3. Protein in sample buffer was loaded into the well and gel was run at a constant voltage of 150V for 2 hours in a Bio-Rad (Mini-PROTEAN Tetra System).

2) Second Dimension

The lane containing protein in the first dimension gel was cut out. The strip was incubated in dissociating solution [1% SDS and 1% 2-mercaptoethanol (Fisher Scientific, New Jersey, USA)] for 1 h. Remove the dissociating solution by using filter paper and glass plate. Post polymerization, the stacking gel was poured around the first dimension strip to make a bridge to the separation gel. Gel was run at a constant voltage of 150V for 100-120 min as the same conditions of one dimensional SDS gel (Nijtmans et al., 2002).

2.5 Gelatin zymography

10% acrylamide gelatin gel was prepared according to the standard procedure. Gelatin stock solution (Sigma Aldrich Co., ST. Louis, USA) was added to running gel [10 mg/ml in H_2O] to get the gelatin concentration of 0.1% (1 mg/ml). Protein sample was mixed with one part 2X sample buffer [2.5 ml of 0.5 M Tris-HCl, pH 6.8, 2.0 ml of glycerol, 4.0 ml of 10% (w/v) SDS, 0.5 ml of 0.1% bromophenol Blue, 10 ml of dH2O] for 10 min at RT and no heat. 20 µg of protein sample was loaded and gel was ran with 1X running buffer [2.9 g of Tris base, 14.4 g of glycine, 1.0 g SDS in 1L dH2O, pH 8.3] at the constant voltage of 125 V for 60 – 120 min in a Bio-Rad minigel system. After running, gel was incubated in 100 ml of 10X Zymogram renaturing buffer [Triton X-100, 25% (v/v) in water (1/9) in deionized water] on a rotary shaker for 30 min at RT. The zymogram renaturing buffer was replaced with 100 ml of 1X zymogram developing buffer [50 mM of Tris base, 0.2 M of NaCl, 5 mM of CaCl2, 0.02% of Brij 35] in addition to these components in developing buffer 5 mM of Zn was added and gel was incubated at rotary shaker for 30 min at RT. Then, the 1X zymogram developing buffer was replaced with the fresh 1X zymogram developing buffer as well as, gel was incubated at 37ºC for at least 4 h and for maximum sensitivity gel was incubated for overnight. Gel was stained with 0.5% (w/v) Coomassie Blue R-250 for 30 minutes and gel then destained with fixing solution [10% v/v acetic acid, 50% v/v methanol, and dH_2O] until the clear bands of protease activity appeared against the dark blue background when protease has broken the substrate (gelatin) (Liota and Stetler-Stevenson, 1990).

2.6 Processing Protein bands for mass spectrometry

Carefully, cut the band of interest from a 1D gel or the protein spot from a 2D gel followed the protocol of in-gel digestion (Speicher et al., 2000). After bands or spots were excised from acrylamide gels, gel pieces were placed in Eppendorf tube. Then, gel pieces were covered with 200 ml of 200 mM ammonium bicarbonate with 40% acetonitrile, incubated at 37 °C for 30 minutes and solution was removed. Moreover, this step was repeated more one time and gel pieces were dried in a Speed Vac concentrator for 15-30 minutes. As well as, 20 µl (0.4 mg of trypsin) of the trypsin solution was added. Furthermore, 50 ml of 40 mM ammonium bicarbonate in 9% acetonitrile solution was added to the gel sample and confirmed that the gel pieces were at the bottom of the tube and covered with liquid. Gel pieces were incubated for 4 hours to overnight at 37 °C. After the incubation, the liquid from gel pieces was removed and transferred to a new labeled tube. This solution contained the extracted tryptic peptides. Extracted peptides were sent to Western University (Functional Proteomics Facility, London Regional Proteomics Centre, Department of Biochemistry) for analysis.

2.7 TCA protein precipitation

Add 1 volume of 100% (w/v) Trichloroacetic acid (TCA) (BDH Laboratory Supplies, England) to 4 volumes of protein sample and incubated 10 minutes at 4°C. After that the sample was pelleted at 14K rpm in a micro centrifuge for 5 min and supernatant discarded. The pellet was subsequently washed two times with 200µl cold acetone. Finally the pellet was dried at 95°C in a heat block for 5-10 min to drive off acetone. For SDS-PAGE, 2X sample buffer was added with β–ME and sample was boiled for10 min in 95°C heat block before loading onto polyacrylamide gel. (Sanchez, L., 2001).

2.8 Anion Exchange Separation of Protein

Anion exchange separation was performed according to a BioLogic Chromatography System protocol (Catalog Number 750-0135) from BIO-RAD. The column (75 x 7.5 mm) was the Bio-Gel© SP-5-PW. A gradient of 0-0.5 M NaCl in 25 mM Tris buffer, pH 7.4, was used over a total volume of 50 ml. A total of 50 fractions (1.0 ml each) were collected and analyzed for gelatinase activity using gelatin zymography.

3. Results

3.1 *M. tuberculosis* genome screening for Proteases

I have compiled below a table (Table 5) listing all the *M. tuberculosis* genes that code for known or putative proteases. The data comes from published work, in-house screening of the annotated *M. tuberculosis* genome (*M. tuberculosis* database -.http://tuberculist.epfl.ch/), and in-house screening of the *M. tuberculosis* genome for genes coding for the consensus zinc-binding sequence of metalloproteases. The gene products from the *in silico* analysis were then analysed for having a secretion sequence by two programs: SignalP and ExProt. 4000 gene products from the *in silico* analysis were screened for having a secretion sequence. More information was then added to the table for each gene product, such as relevant literature and findings on their role or function. For those genes with little published information, I did sequence homology searches to try and find possible functions for them using BLAST. Finally, I eliminated some proteases from this list by considering their function (lacking the zinc binding signature motif) or their localization (those that are lipid-anchored or lipoproteins were excluded or missing the secretion signal peptide). This dramatically reduced the list of "interesting" gene products to the following: Rv3207c. (Table 5) (Appendix section).

3.2 Gelatinase activity in the supernatant of *M. bovis* BCG

Preliminary experiments focussed on an assessment of the gelatinase activities present in the supernatant of *M. bovis* BCG cells grown in Sauton's medium with two different sources for nitrogen by zymography. One activity was detected in both supernatants that have ammonium chloride (AC) and asparagine (AS) as a nitrogen source. This activity had a molecular weight of approximately at 80-85 kDa indicating that *M. bovis* BCG does, in fact, secrete at least one protease (Figure 3).

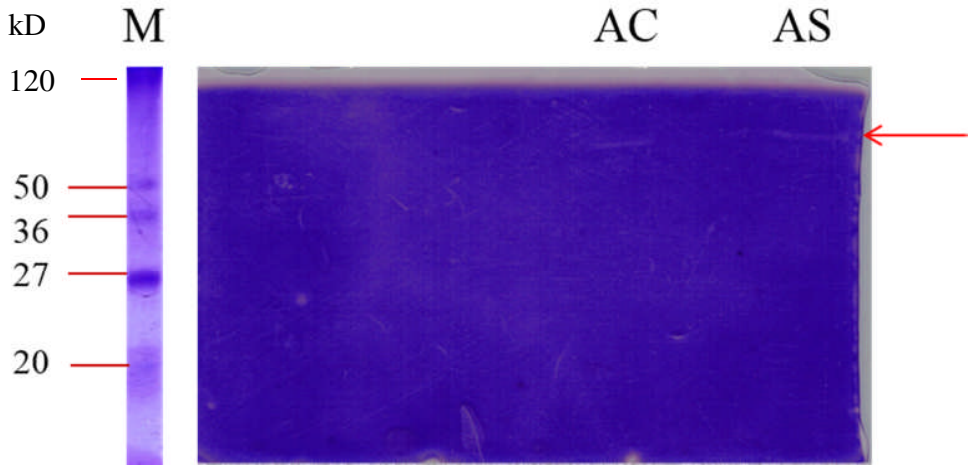


Figure 3: Zymogram comparing gelatinase activity in the culture supernatant of M. bovis BCG grown in Sauton's medium with two different sources of nitrogen**.** AC = supernatant of *M. bovis* growing in Sauton's media (ammonium chloride) - acidic, AS = supernatant of *M. bovis* growing in Sauton's media (asparagine) – alkaline. Culture media from *M. bovis* BCG grown with different nitrogen sources were purified from the bacteria concentrated and run on a 12% SDS gel supplemented with 10% gelatin. The gel was stained with coomassie Brilliant blue and the presence of clear areas in the gel indicates protease activity (red arrow)

The indicated band which corresponds to the gelatinase activity in AS media appears to have a higher molecular weight than the activity in AC media. The varying molecular weights could represent two different proteases (depending upon the nitrogen source. Alternatively, the lower molecular weight protease may stem from the higher molecular weight protease (cleavage product).

3.3 Analysis of Mass spectrometry results

I) First trial:

Based on the detected activity from the zymogram (Figure 3) the purified concentrated culture medium from *M. bovis* was run on a denaturing 12% SDS PAGE gel and stained with Coomassie Brilliant blue. Bands corresponding to the zymogram were excised from the gel and digested with trypsin for protein identification by mass spectrometry, with the caveat that the molecular weight for the protease(s) under non-denaturing conditions (zymogram) could be different than under denaturing conditions (SDS PAGE gel) and that the protein having protease activity identified on the zymogram may not exhibit any or strong Coomassie Blue staining (Figure 4). Seven candidate proteins were identified from the bands excised from the gel (as shown in Table 1).

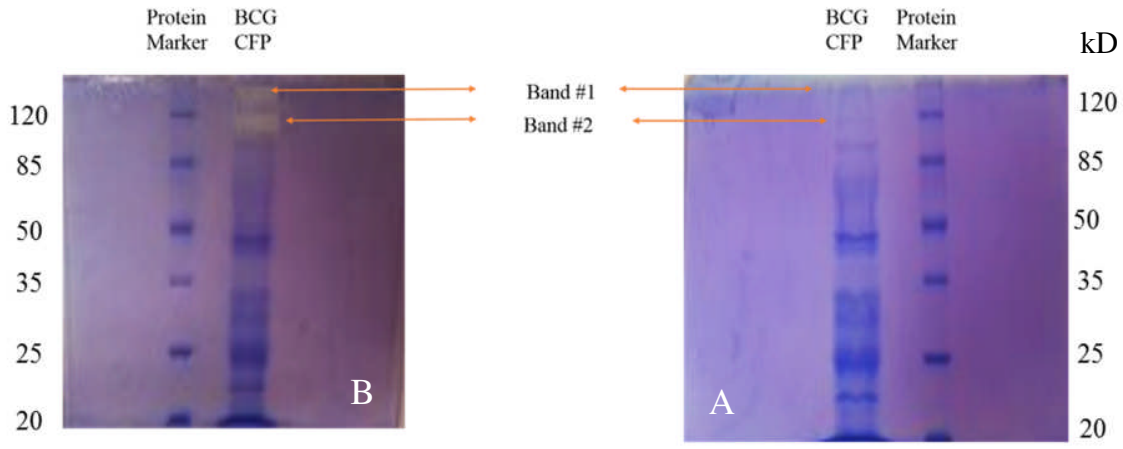


Figure 4: Excision of candidate protease purified from culture media from *M. bovis* for mass spectrometric identification. (A) 12% SDS PAGE gel shows bands of culture filtrate protein of *M. bovis* BCG. (B) 12% SDS PAGE gel shows the area of excised bands (red arrows).

Table 1: Mass Spectrometry identification of excised proteins purified from the culture media from *M. bovis*.

Sample ID/*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
BCG Band#1	MASCOT	125	Uncharacterized protein Rv2074	45%	22	-
		125	hypothetical protein	28%	16	-
		125	Phytoene synthase	26%	20	-
		125	ATP-dependent Clp protease adapter protein ClpS	13%	7	-
BCG Band#2	MASCOT	118	hypothetical protein	22%	19	-
		118	hypothetical protein Rv1489c	12%	7	-
		118	membrane protein	9%	9	-

II) Second trial: attempt to identify zinc dependent protease

In a second attempt to isolate and characterize zinc-dependent activities, proteins from culture media of *M. bovis* cells were first resolved on a native gel. The entire lane was excised from the gel and placed horizontally on a 12% SDS-PAGE gel containing 10% gelatin. The SDS gel was developed with a zinc containing solution and stained with Coomassie brilliant blue. A clear spot of protease activity was detected (Figure 5A). To identify the protein responsible for the activity the process was repeated except the SDS gel contained no gelatin and was not developed for activity only stained with Coomassie Brilliant blue (Figure 5B), assuming that the strongest staining spot in that region of the gel was the desired protease.

Mass spectrometry analysis identified 16 possible proteins in the excised region of the 2D gel (listed in Table2).

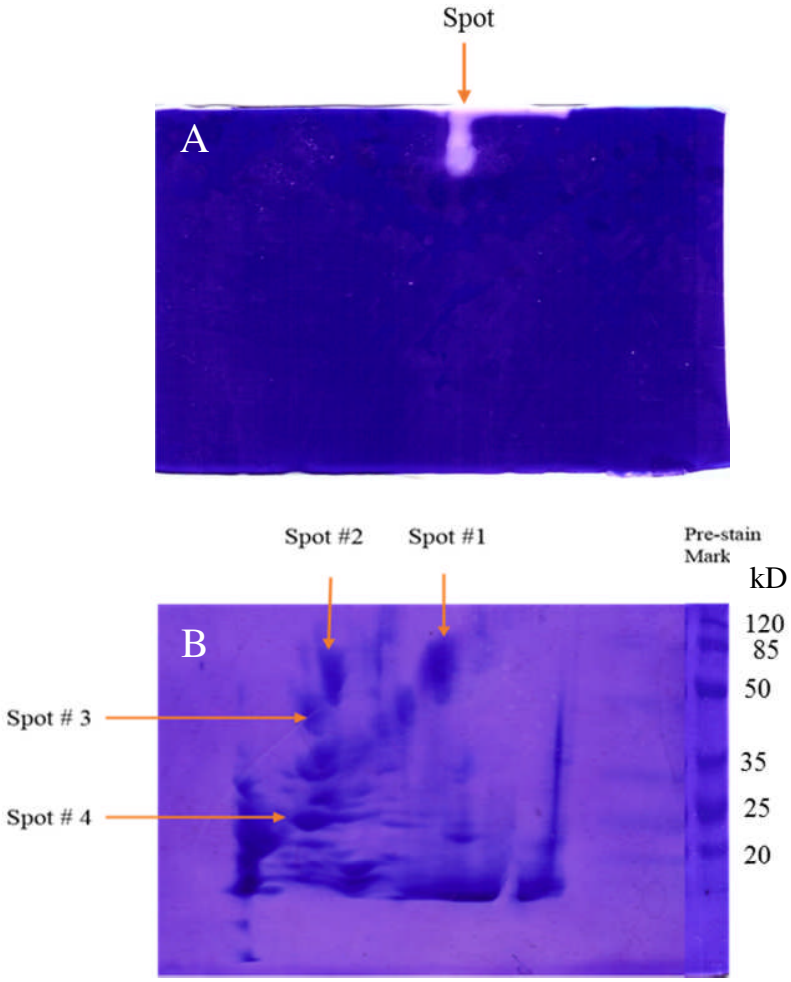


Figure 5: Zymography of supernatant from *M.bovis* BCG for protease identification.(A) 2-D gel (12% zymography gel) shows a spot that has gelatinase activity which indicates there is protease in the supernatant of *M. bovis* BCG. (B) 2-D gel (12% SDS gel) shows all protein spots that included in the supernatant. Red arrows indicate spots were cut and digested by trypsin for identification by mass spectrometry.

Table 2: Protein identification of excised spots through mass spectrometry

Sample ID/*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
BCG Spot#1	MASCOT	90	Glycerol-3-phosphate acyltransferase	19%	32	-
	Protein Prospector	90	Probable ATP-depended Clp protease ATP-binding subunits	6.4%	196	63261
BCG Spot#2	MASCOT	86	Antitoxin Rv0298	53%	28	-
		86	PE-PGRS family protein, partial	49%	29	-
		86	Uncharacterized protein Rv1355c	14%	37	-
	Protein Prospector	86	ATP-depended DNA helicase RecG	6.3%	419	330584
		86	Uncharacterized protein Rv2030c	5.9%	229	505360
		86	Cation-transporting P-type ATPase A	5.3%	56.7	78021
BCG Spot#3	MASCOT	60	Glycine--tRNA ligase	13%	21	-
		60	ATP-dependent Clp protease ATP-binding subunit ClpX	4%	14	-
	Protein	60	Chaperone protein	7.1%	222	98789

	Prospector		Dnak			
BCG Spot#4	Mascot	30	hypothetical protein	38%	44	-
		30	Uncharacterized protein Rv3466	24%	27	-
		30	Uncharacterized protein Rv1588c	21%	22	-
		30	Probable thioesterase TesA	14%	20	-
	Protein Prospector	30	Ribonuclease Z	14%	1393	377614

III) Third trial: protein precipitation for the identification of zinc dependent proteases from *M. bovis*

In a third attempt to identify zinc-dependent protease activities, the cultures AS media proteins were precipitated with Trichloroacetic acid (TCA). The precipitated proteins were re-suspended in buffer and loaded equally in each lane on 10%SDS gel. Bands that matched those of the zymogram (Figure 3) were excised and processed for mass spectrometry (Figure 6).

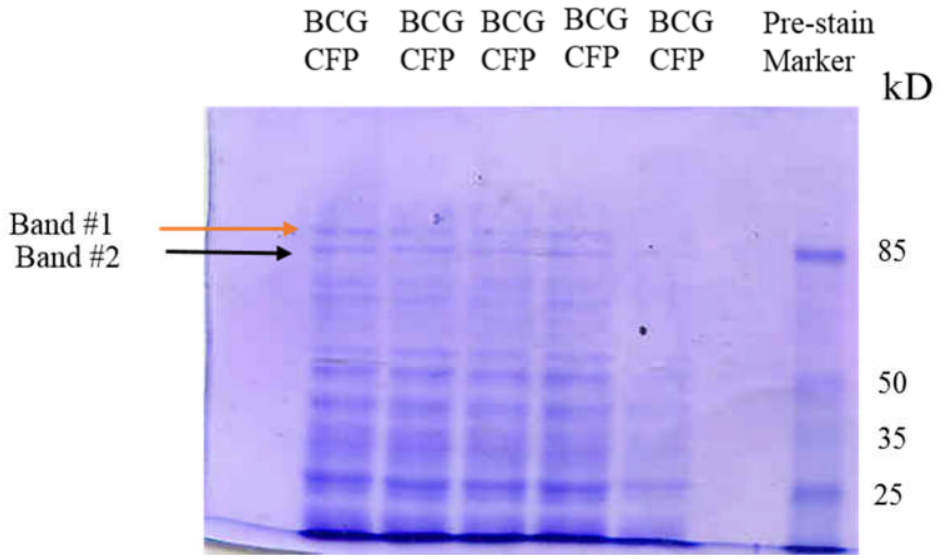


Figure 6: TCA precipitation from *M.bovis* BCG supernatant for protease identification. 10% SDS gel shows BCG CFP (Culture Filtrate Proteins) after TCA (Trichloroacetic acid) precipitation. Each lane has an upper first and second band located at the same site of the bands on the zymography gel. Those bands will be cut and digested by trypsin and identified by mass-spectrometry.

Table 3: Protein identification of excised bands from TCA precipitated culture media

Sample ID/*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
BCG Band#1	MASCOT	100	Putative antitoxin VapB36	61%	28	-
		100	PE-PGRS family protein	45%	43	-
		100	Probable helicase HelY	24%	44	-
		100	Replicative DNA helicase	20%	22	-
		100	ATP-dependent helicase	18%	57	-
	Protein Prospector	100	Serine/threonine-protein kinase PknK	11.4%	11190	293790
		100	Glutamate-ammonia-ligase adenylyltransferase	9.9%	10716	148052
		100	Exodeoxyribonuclease V gamma chain	8.7%	6227	118156
		100	UPF0182 protein Rv0064/MT0070	9.5%	2793	496589
		100	Probable ATP-depended Clp protease ATP –binding subunit	9.1%	2120	63261

BCG Band#2	MASCOT	85	Glycerol-3-phosphate acyltransferas	21%	32	-
		85	Putative transferase	15%	32	-
	Protein Prospector	85	Protein translocase subunit SecA 2	13.7%	993	416631

IV) Fourth trial: Post anionic exchange chromatography

The proteins in the culture supernatant were separated by anion exchange chromatography to try to collect proteins with similar charge profiles in fractions collected from the column. Protein fractions with readings above 0.25µg/ml at 280nm absorbance were pooled. They were chosen to run on a12% zymography gel to find which fractions have gelatinase activity. Next, a 12% SDS gel was run and compared between the gels based on an approximate band size. The equivalent Commassie-Blue stained protein was then cut from the gel, digested with trypsin, and subjected to mass spectrometry to identify proteins associated with the stained band.

In this trial, there were 45 protein fractions which were pooled together in 5 fractions aliquots because each individual fraction had low content of protein that will make the detection of the activity through a zymogram gel difficult. We found that the first pooled fraction (fractions 1 to 5) was the only fraction having gelatinase activity. Next this fraction was run on a 12% zymogram gel (Figure 7 A) as well as a 12% SDS gel (Figure 7 B) to find an approximate size of band which is matching with the band size in the zymogram gel. This band was cut and digested by trypsin to identify by mass spectrometry. Only one candidate protein was identified in the mass spectrometric analysis (Table 4).

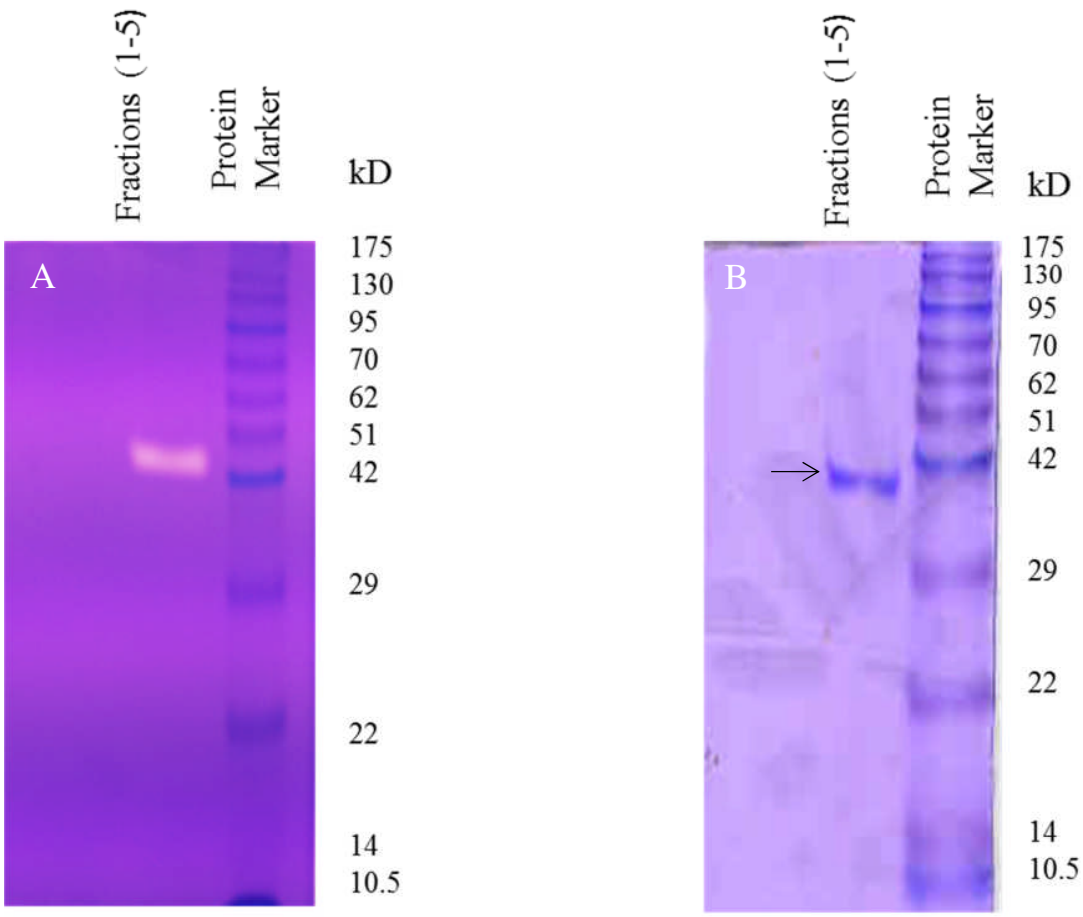


Figure 7: Identification of protease activity from anionic exchange chromatography. (A) 12% zymogram gel includes pooled fractions 1-5 which show a band of approximately 45 kDa. (B) 12% SDS gel resolving pooled fractions 1-5 shows a band of approximately 40 kDa and this band was excised and processed for mass-spectrometry.

Table 4: Protein identification for post anionic exchange chromatography (fractions 1-5)

Sample ID/*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
Fractions 1-5	Protein Prospector	40	hypothetical protein	12.2%	9.80	13696101

V) Fifth trial: N-terminal sequncing

In yet another attempt, post transfer from the 12% SDS gel to PVDF membrane, we cut the same area in the membrane which corresponded to the band shown in Figure 7 B. We then sent the exciesd PVDF membrane for N-terminal sequencing (Figure 8). The resulting N-terminal sequence was VTGGGA. Using P-BLAST on the NCBI website, several candidate proteins were identified. The rest of the findings from this analysis are listed in the appendix section. The best candidate for this sequence was a protein called PstS3, which is involved in phosphate transport. It has the following sequence:

PstS3 PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS3 (PBP-3) (PSTS3) (PHOS1) 1035368:1036480 forward MW: 37953

MKLNRFGAAVGVLAAGALVLSACGNDDNVTGGGATTGQASAKVDCGGKKTLKASGS
TAQANAMTRFVNVFEQACPGQTLNYTANGSGAGISEFNGNQTDFGGSDVPLSKDEAAA
AQRRCGSPAWNLPVVFGPIAVTYNLNSVSSLNLDGPTLAKIFNGSITQWNNPAIQALNR
DFTLPGERIHVVFRSDESGTTDNFQRYLQAASNGAWGKGAGKSFQGGVGEGARGNDG
TSAAAKNTPGSITYNEWSFAQAQHLTMANIVTSAGGDPVAITIDSVGQTIAGATISGVGN
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YIPIPDEFKSRLSTAVNAIA

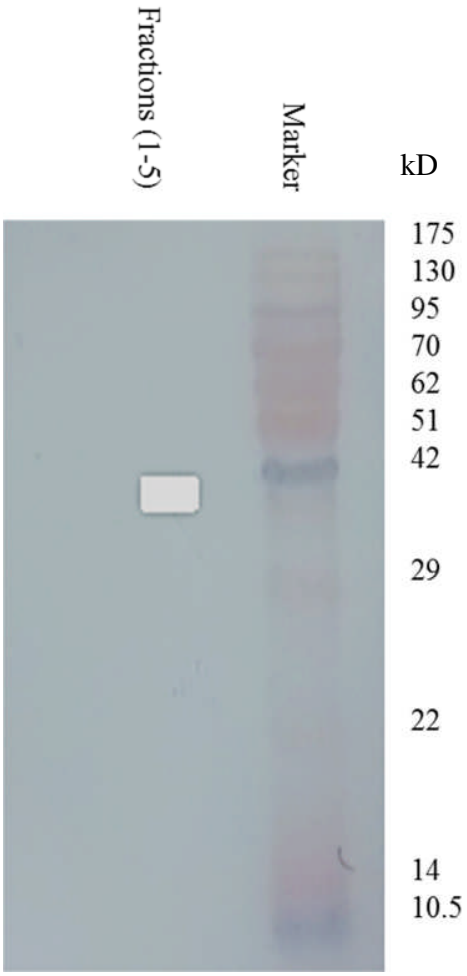

Figure 8: PVDF membrane includes transferred gel and excised area which was sent for N-terminal sequencing.

4. Discussion

Mycobacterium tuberculosis is a facultative intracellular pathogen, known to play a major role in the etiology of tuberculosis. Tuberculosis is a serious and potentially life-threatening illness affecting approximately 33% of the world's population. The phagosome maturation process, which concludes in the formation of the phago-lysosome, is a crucial step for successful clearance of the pathogen in humans by macrophages; however, *M. tuberculosis* escapes macrophage killing by inhibiting phagosome maturation (Vergne, 2003). Thus, a full understanding of the mechanism that mediates the survival of the pathogen in phagocytic cells is essential. The *M. tuberculosis* zinc-dependent metalloprotease Zmp1 has been proposed to play a key role in the inhibition of phagosome maturation by suppressing inflammasome activation through inhibition of caspase-1 activation. This prevents processing of pro-IL-1β into IL-1β and blocks phagosome maturation (Master et al., 2008). When caspase-1 is activated, this cleaves pro-IL-1 β into IL-1β, which is secreted in an autocrine and paracrine fashion to promote phagosome fusion with intracellular lysosomes and the early inflammatory response (Sakamoto, 2012; Fantuzzi & Dinarello, 1996). In the study of Master et al (2008) they used macrophages infected by a *M. tuberculosis* Zmp1 mutant to demonstrate that Zmp1 is required for *M. tuberculosis* virulence and survival in the macrophage. Also, they found Zmp1 in the whole cell lysates of *M. tuberculosis*. All these findings came from a genetic inactivation of Zmp1 gene in *M. tuberculosis* but the details of how the Zmp1 gene inactivation affects pathogenesis is still unknown. Therefore, further studies are required to understand the role of the Zmp1 gene product (a Zn-metalloprotease) in the pathogenesis of *M. tuberculosis*. The focus of my thesis was the identification of secreted proteases in *M. bovis* that may could play a role in phagosome maturation or inflammasome inactivation. To achieve this objective, we first screened the *M.*

tuberculosis genome *in silico* for Zn^{++}-dependent secreted proteases that may play a role in blocking phagosome-lysosome fusion. Genes had to code for secreted proteins that were known proteases, had homology to known proteases, and/or contained the consensus sequence for the Zn^{++} binding site of Zn^{++}-dependent proteases. The proteins in spent medium from *M. bovis* BCG cultures were collected to assess for the presence of one or more of the above proteases using a gelatinase activity test (zymography). Bands associated with the gelatinase activity were then purified and subjected to mass spectrometry and N-terminal sequencing in order to confirm that *M. bovis* BCG secrets one or more metalloproteases and to identify these proteases.

Based on the analysis presented in this thesis, there are sixty six candidate genes within the *M. tuberculosis* genome which may encode for a secreted protease (Table 5). As expected, some of them have signal peptides and zinc binding consensus such as:

- Rv3207c, which is 31kD size, is known as conserved hypothetical protein. It was identified by mass spectrometry in the culture filtrate of *M. tuberculosis* H37Rv but not the membrane protein fraction or whole cell lysates (de Souza et al., 2011).

Other candidate proteins having zinc binding consensus sequences, with no signal peptides are:

- Rv3610 (FtsH), which is 81kD in size, is involved in cell division, and is thought to act as ATP-dependent zinc metalloprotease (Srinivasan et al., 2006, Ribeiro- Guimarães et al., 2007). It is believed to have a regulatory role in stress response and in the secretion of specific proteins during the host's adaption to its environment (Ribeiro- Guimarães et al., 2007).

- Rv0563 (htpX), which is a protease transmembrane protein and probable heat shock protein and is 30kD in size. It also is possibly involved in adaption to the host environment and hydrolysis of specific peptides and proteins (Malen et al., 2007).
- Rv1977, which is 38kD in size, and possibly acts as zinc-dependant enzyme with a chaperone function. It is not involved in virulence because it is deleted in *M. bovis* (Forrellad et al, .2013).
- Rv0198c (Zmp1), which is 37kD size, is crucial for the survival of *M. tuberculosis* and *M. bovis* BCG in murine J774 macrophages. It also plays a role in the virulence of *M. tuberculosis* in mice (Master et al, 2008).

For those candidate genes that have no clear roles or functions, BLAST searches were conducted to identify homologous genes, whose activities and properties are known. This could help identify possible functions for them. For instance:

- Rv2575 is 30kD in size and is found in *Brucella melitensis* as a neutral zinc metalloprotease. It is also found in *M. tuberculosis* and is characterized as a conserved glycine rich membrane protein. (from the *M.tuberculosis* database- http://tuberculist.epfl.ch/) (DelVecchio et al., 2002).

- Rv0125 (PepA) is a 34kD protein identified in filtrates of *M. tuberculosis* H37Rv cultures by 2-DE, combined with MALDI-TOF MS and LC coupled MS/MS (Malen et al, .2007). In *M. tuberculosis* it is identified as a serine protease (from the *M.tuberculosis* database - .http://tuberculist.epfl.ch/). It is found in *Bacillus thuringiensis* as a metalloprotease produced during the early stage of sporulation (Li & Yousten, 1975).

- Rv3668c is 23kD in size and found in *C. diphtheriae* as a putative protease (ExPasy (http://www.expasy.org/blast). Rv3668c is also identified as a protease in the *M. tuberculosis* database (.http://tuberculist.epfl.ch/).

The remaining candidate secreted proteases have unknown functions, and no comments about their properties can be made because no current data is available (to my knowledge).

In this study, my approach was to grow *M. bovis* BCG cells in a modified growth medium (protein-free Sauton's medium) to mid-/late-log phase, harvest the culture, and remove the bacteria by filtration. The spent medium (free of bacterial cells, ie. the culture supernatant) was then used as the source of secreted proteins for further analysis. The proteins in the culture supernatant were then separated using one- and two-dimensional SDS-polyacrylamide gel electrophoresis. The position of the proteases in the one and 2-D gels were identified by zymography, using gelatin as a substrate. All zymogram gels in this study were prepared in non-reducing conditions (Chung et al., 2011). Also, there is no boiling of the samples because heat denatures proteins and may eliminate enzyme activity (Kleiner & Stetler-Stevenson, 1994).

I conducted several zymography experiments using one and 2D gels in order to identify bands or spots representing protease activities that could be excised from the gels, digested with trypsin, and subsequently identified by mass-spectrometry. Protein identification by mass-spectrometry revealed various hits in all trials and those hits were analyzed by two programs found in ExPasy SIB Bioinformatics Resource Portal: Mascot and Protein Prospector. Both of these programs are available at http://www.expasy.org/. These hits have an estimated molecular mass that may or may not match with the original band or spot size which was excised from the gels due to various possible post-translational modifications. Nevertheless, attempts were made to find the closest hit (stained band) that matched with the size of gelatinase activity.

Disappointingly, most of the hits in all trials were hypothetical proteins; however some of them may prove to be proteases that play a role in the growth and survival of *M. tuberculosis*.

4.1 Trial 1:

In this trial (Figure 4), 12% SDS gels were used to identify bands that matched the protease activity band that appeared on the 12% zymogram gel (Figure 3). The band selected on the SDS gel was based on the strongest Coomassie Blue staining band that corresponded to the approximate region of the band (clearing) on the zymogram gel. The first band was located at approximately 125 kDa and the second band at 90 kDa. The majority of the proteins identified in this trial were previously uncharacterized proteins, hypothetical proteins, membrane proteins, and one relevant and previously characterized protein (the ATP-dependent Clp protease adapter protein ClpS) (Table 1). Unfortunately, this protein is unrelated to a Zn-dependent metalloprotease. This may be because of several reasons: for example, the identified bands were located at significantly different molecular weights than the band of protease activity on the zymogram, which means that these are different proteins, or the protease activity did not stain with Coomassie Blue and did not appear on the stained SDS gel. In the future, it may be best to use a more sensitive protein stain to identify the band likely corresponding to the gelatinase activity.

4.2 Trial 2:

Four spots were excised from a 12% 2-D gel, based on a comparison with the 12% 2-D zymogram gel and identified through mass spectrometry (Figures 5 A and B). The spots were chosen based on the strongest staining of spots with Coomassie that corresponded to the approximate area of the zymogram spot. The first spot, which was located at 90kD, had two hits

obtained from mass spectrometry identification, namely the probable ATP-dependent Clp protease ATP-binding subunit and Glycerol-3-phosphate acyltransferase (Table 2).

The second spot was located at 86 kD and had several hits not related to metalloproteases: Antitoxin Rv0298, PE-PGRS family protein, partial, ATP-depended DNA helicase RecG and Cation-transporting P-type ATPase A (Table 2).

The third spot, located at 60 kD, had many hits that included ATP-dependent Clp protease ATP-binding subunit ClpX , Glycine--tRNA ligase, and the chaperone protein Dnak.

Dnak was found in the lysate of *Mycobacterium bovis* BCG and it has a role in the survival of *Mycobacteria spp.* (Lee et al., 2014). A recent study revealed that Dnak is critical for the survival of *Mycobacterium* species and it is located in the cell wall, cytoplasm, periplasmic space, and plasma membrane (Lee et al., 2014). In addition, it is found in the CFP of *M. bovis* BCG (Berrêdo-Pinho et al., 2011).

The fourth spot was located at 30 kD which had several hits of hypothetical proteins, namely: Probable thioesterase TesA, an uncharacterized protein, and Ribonuclease Z (Table 2).

The third and fourth spots were excised for the purpose of aligning and comparing results with other studies that conduct proteomics using 2-D gels. All of the four identified spots did not show traces that could be associated to Zn-dependent metalloproteases for many reasons. One reason for this may be that the alternative method of 2-D SDS gel and 2-D zymogram gel that was used might have left some proteins precipitated and stuck at the origin of the second dimension gel, resulting in improper resolution. Again, another possible reason is that the identified Coommasie Blue-stained spots that were cut from the 2D gel were different proteins than those that had gelatinase activity on the 2D zymograms.

4.3 Trial 3:

In this trial, the culture supernatant was precipitated using TCA to concentrate the proteins. Post TCA precipitation, sample resuspension and resolution by 10% SDS PAGE, two bands were excised from the 10% SDS gel (Figure 6) and digested by trypsin and identified by mass spectrometry. The first band, located at 100 kD, yielded various hits: a putative antitoxin VapB36, PE-PGRS family protein, a probable helicase HelY, a replicative DNA helicase, ATP-dependent helicase, a serine/threonine-protein kinase PknK, a glutamate-ammonia-ligase adenylyltransferase, an exodeoxyribonuclease V gamma chain, an UPF0182 protein Rv0064/MT0070, and a probable ATP-depended Clp protease ATP-binding subunit (Table 3). The PknK is a large multifunctional protein with a transmembrane domain (Kumar et al., 2009). This kinase, as well as other related kinases, has been implicated in the modulation of growth in culture and during infection (Malhotra et al., 2010). Moreover, various studies have shown that, PknK phosphorylates proteins that involved in cell division and transcription On the other hand, there is limited information on the mechanisms of STPK-mediated regulation of mycobacterial growth (Malhotra et al., 2012). The second band was located at 85 kDa and had several hits, but none belonging to zinc metalloproteases. These included a glycerol-3-phosphate acyltransferase, a putative transferase, and protein translocase subunit SecA2 (Table 3). This trial, attempted to identify the indicated band that matched the approximate area of the protease activity band in the zymography gel. The mass spectrometry identification results showed that these two bands were not related to the target protein for various reasons: the stained bands were again cut from a different position (molecular weight) than the bands of protease activity, and, possibly, the protease activity did not stain with Coomassie and consequently did not appear on SDS gel.

4.4 Trial 4:

We also used anion exchange chromatography to fractionate the proteins excreted by *M bovis* BCG (Alito et al., 2003). Proteins were separated based on the basis of their net charges. In anionic exchange chromatography, the column has positive charge resins which bind negatively charged proteins (GE Healthcare, 2010). Then, the bound proteins are eluted by increasing the concentration of sodium chloride applied to the column. The migration of protein down and out of the column is directly dependent on the negative charge density. For instance, the proteins that have a high density of net positive charge will emerge first. After anionic exchange chromatography, 45 fractions were collected and the fractions were pooled together in groups of five yielding a total of 9 fractions. Furthermore, these fractions were run in a 12% zymogram gel to identify which fraction has gelatinase activity. Only the first pooled fraction was found to have gelatinase activity in the 12% zymogram gel and this fraction was also resolved by a 12% SDS gel (Figures 7 A and B). In the 12% zymogram gel, only one band from fractions (1-5) with gelatinase activity appeared at ~45 kD however, in the 12% SDS gel, it appeared at 41 kD, likely because the strongest staining band on the stained SDS gel was located there, while the gelatinase activity represented a much more weakly expressed protein that does not stain well with Coomassie Blue. Alternatively, the anomalous migration of the protein in the zymogram is due to presence of the gelatinase in the zymogram. Also when the strongly staining band was excised from the 12% SDS gel, digested by trypsin, and identified by mass spectrometry, only one hit was obtained, which was a hypothetical protein (Table 4). In this trial, the identified band did not show any hint of metalloprotease, possibly because the band identified in the 12% SDS PAGE was cut at a different molecular weight than the protease band. An alternative way to purify the right protein would have been to run all the individual fractions and identify which

fractions induce strong clearing on a zymogram, then fractionate on the basis of molecular weight (gel filtration), until a progressively purer fraction of protease activity was obtained that can then be run on an SDS gel, excise, and subjected to mass spectrometry (if there is a sufficient amount of protein).

4.5 Trial 5: N-terminal sequencing:

In the final trial, the pooled fraction sample from trial 4 was run on a SDS PAGE gel and transferred to a PVDF membrane. The area of the membrane corresponding to the region of protease activity on the previously run zymogram gel, was cut out and sent for N-terminal sequencing. Six N-terminal residues were found in the proteins excised from the PVDF membrane containing a band near the gelatinase activity after anion exchange chromatography (appendix section). There were (S-Ser: G-Gly; V-Val), T-Thr, G-Gly, G-Gly, G-Gly, A-Ala. Thus possible N-terminal sequences would include STGGGA, GTGGGA, or VTGGGA. Blasting these sequences against the *M. tuberculosis* translated open reading frames (ORFs) database found that VTGGGA is the best sequence matching a known N-terminus of *M. tuberculosis* proteins. Furthermore, this sequence revealed several candidates proteins (appendix section). The best candidate for this sequence is a protein called PstS3 which is involved in phosphate transport. It is also required for survival in macrophages (Rengarajan et al., 2005).

PstS3 was considered the best candidate, as the size is close to the actual size of the band (Figure 7 B). The protein also contained the whole sequence, as opposed to the other hits, which only had a part of the sequence.

In my opinion, all four trials conducted in this thesis identified different proteins than the target. These results can be explained by many possibilities: missing the right protein by cutting the band or spot with the strongest Commassie Blue staining near but not right at the area of gelatinase activity. In addition, the *M. bovis* BCG might secret the metalloprotease under specific stress condition, the secretion of metalloprotease enzyme could be below the detection limit of the Coomassie stain, and *M. bovis* BCG might secret metalloproteases but distinguishing these proteins from other proteins on Commassie Blue stained gels was not possible.

For possible future approaches, an alternative must be investigated to isolate and identify a secreted gelatinase. This could involve cloning of pstS3 to see if it has gelatinase activity through zymography. Also, there is another method which involves cutting the band that shows gelatinase activity and running it on an SDS gel and identifying a band that will appear after electrophoresis. This would require a large amount of culture supernatant and running a large gel that will help to provide an appropriate amount of protein to identify through mass-spectrometry.

5. Conclusion

The goal of this study was to confirm that *M. bovis* BCG secretes one or more metalloproteases and to identify those present in the culture supernatant. Sixty six gene products resulted from screening of the *M. tuberculosis* genome that could encode for a secreted protease. Several attempts were made to identify metalloproteases in the culture supernatant, such as comparing between one or 2-D SDS gels and one or 2-D zymogram gels, then excising the corresponding bands or spots and digesting them with trypsin for possible identification by mass-spectrometry, an anionic exchange chromatography, or N-terminal sequencing. Most of the hits identified by mass-spectrometry were hypothetical proteins and probable proteins which are likely unrelated to metalloproteases. Gelatinase activity was found in the first five pooled fractions through anionic exchange chromatography. This pooled fraction band appeared approximately at 45kD on the Zymogram but the nearest Commassie Blue staining band on SDS gels was at ~41 kD. The 41 kD band which was transferred to PVDF membrane for N-terminal sequencing. This approach identified PstS3, which has a mass of 37 kD and is involved in phosphate transport. Currently, there is no published evidence that indicates that pstS3 is a protease. This study clearly identifies a protease (gelatinase) of 45 kD, which is secreted from *M. bovis* BCG cells. However, the approaches currently used to date to identify this protease activity have not been successful. All results support the hypothesis but not fully since the identification of protease was not revealed.

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Appendix

Blast result of N-terminal sequencing for unidentified protease

Wellcome Trust Sanger Institute (Hinxton, UK)
http://www.sanger.ac.uk/cgi-bin/blast/submitblast/mycobacterium

>pstS3 PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS3 (PBP-3) (PSTS3) (PHOS1)
1035368:1036480 forward MW:37953
[Full Sequence] Length = 370

Score = 35 (17.4 bits), Expect = 15., P = 1.00000
Identities = 7/9 (77%), Positives = 7/9 (77%)
[HSP Sequence]

Query: 9 DREVTGGGA 17
D VTGGGA
Sbjct: 26 DDNVTGGGA 34

>pstS3 PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS3 (PBP-3) (PSTS3) (PHOS1) 1035368:1036480 forward MW:37953
MKLNRFGAAVGVLAAGALVLSACGNDDNVTGGGATTGQASAKVDCGGKKTLKASGS
TAQANAMTRFVNVFEQACPGQTLNYTANGSGAGISEFNGNQTDFGGSDVPLSKDEAAA
AQRRCGSPAWNLPVVFGPIAVTYNLNSVSSLNLDGPTLAKIFNGSITQWNNPAIQALNR
DFTLPGERIHVVFRSDESGTTDNFQRYLQAASNGAWGKGAGKSFQGGVGEGARGNDG
TSAAAKNTPGSITYNEWSFAQAQHLTMANIVTSAGGDPVAITIDSVGQTIAGATISGVGN
DLVLDTDSFYRPKRPGSYPIVLATYEIVCSKYPDSQVGTAVKAFLQSTIGAGQSGLGDNG
YIPIPDEFKSRLSTAVNAIA
>Mb2332c HYPOTHETICAL GLYCINE RICH PROTEIN 2557099:2557530
reverse MW:14585
[Full Sequence] Length = 143

Score = 22 (12.8 bits), Expect = 21., Sum P(2) = 1.00000
Identities = 4/4 (100%), Positives = 4/4 (100%)
[HSP Sequence]

Query: 14 GGGA 17
GGGA
Sbjct: 140 GGGA 143

>thiD PROBABLE PHOSPHOMETHYLPYRIMIDINE KINASE THID (HMP-PHOSPHATE KINASE)

(HMP-P KINASE) 508777:509574 reverse MW:27510
[Full Sequence] Length = 265

Score = 23 (13.2 bits), Expect = 2.0e+02, P = 1.00000
Identities = 4/5 (80%), Positives = 5/5 (100%)
[HSP Sequence]

Query: 13 TGGGA 17
+GGGA
Sbjct: 14 SGGGA 18

>Mb3634c PROBABLE CONSERVED TRANSMEMBRANE PROTEIN RICH IN ALANINE AND ARGININE
AND PROLINE 3989544:3990737 reverse MW:43016
[Full Sequence] Length = 397

Score = 26 (14.2 bits), Expect = 1.8e+02, Sum P(2) = 1.00000
Identities = 5/6 (83%), Positives = 5/6 (83%)
[HSP Sequence]

Query: 11 EVTGGG 16
E TGGG
Sbjct: 385 ESTGGG 390

>Mb3341c SECRETED PROTEIN ANTIGEN 3655230:3655541 reverse MW:10767
[Full Sequence] Length = 103

Score = 22 (12.8 bits), Expect = 9.6, Sum P(2) = 0.99994
Identities = 4/4 (100%), Positives = 4/4 (100%)
[HSP Sequence]

Query: 14 GGGA 17
GGGA
Sbjct: 100 GGGA 103

blast server results

Retrieve result for id:
Format:

Your BLAST query has been added to the queue of jobs.
The majority of BLASTs are completed within two minutes.

To retrieve your results, click the retrieve button above, or use the following URL: http://www.sanger.ac.uk/cgi-bin/blast/getblast?id=d02J9601d7992y89q79F7;format=graphic
Click here to start a new blast job

Options: cpus=1 -warnings S=10 B=100 -filter=seg V=100
Job id d02J9601d7992y89q79F7.1 status is DONE

BLASTP 2.0MP-WashU [04-May-2006] [linux26-x64-I32LPF64 2006-05-10T17:22:28]

Reference: Gish, W. (1996-2006) http://blast.wustl.edu

Query= UNKNOWN-QUERY
(17 letters)

Database: /corebio/data/blastdb_web/yeastpub/MB.pep
3920 sequences; 1,307,749 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

Sequences producing High-scoring Segment Pairs:	Smallest Sum High Score	Probability P(N)	N
Mb3522c CONSERVED HYPOTHETICAL MCE ASSOCIATED PROTEIN 385...	42	0.58	1
TB18.6 CONSERVED HYPOTHETICAL PROTEIN TB18.6 2380196:2380...	26	0.995	2
Mb0967 HYPOTHETICAL PROTEIN 1052827:1053105 forward MW:9737	18	0.997	3
Mb0782c CONSERVED HYPOTHETICAL PROTEIN 856015:856347 reve...	32	0.998	1
PPE21 PPE FAMILY PROTEIN 1735310:1737346 reverse MW:66651	41	0.999	1
Mb3951c HYPOTHETICAL PROTEIN SIMILAR TO JAG PROTEIN 43422...	34	0.9997	1
Mb0623 HYPOTHETICAL PROTEIN 704058:704444 forward MW:14134	28	0.9999	2
Mb3341c SECRETED PROTEIN ANTIGEN 3655230:3655541 reverse ...	22	0.99994	2
Mb1658c HYPOTHETICAL PROTEIN 1821753:1822196 reverse MW:1...	32	0.99995	1
Mb1412c PUTATIVE TRANSFERASE 1547258:1547896 reverse MW:2...	25	0.99997	2
Mb1749c CONSERVED HYPOTHETICAL PROTEIN 1932370:1932759 re...	31	0.99997	1
Mb1293c HYPOTHETICAL HIT-LIKE PROTEIN 1408174:1408608 rev...	27	1.00000	2
whiB3 TRANSCRIPTIONAL REGULATORY PROTEIN WHIB-LIKE WHIB3 ...	26	1.00000	2
Mb3073c HYPOTHETICAL PROTEIN 3364582:3364866 reverse MW:1...	20	1.00000	2
wag22a PE-PGRS FAMILY PROTEIN WAG22A [FIRST PART] 1983133...	28	1.00000	1
Mb0850c PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN 92157...	18	1.00000	3
PE_PGRS29 PE-PGRS FAMILY PROTEIN 1652355:1653467 reverse ...	35	1.00000	1
pstS3 PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS3 (PB...	35	1.00000	1
Mb0991c CONSERVED HYPOTHETICAL PROTEIN 1077672:1078274 re...	32	1.00000	2
Mb2286 Possible oxidoreductase 2514595:2515548 forward MW...	34	1.00000	1
Mb3084c POSSIBLE TRANSCRIPTIONAL REGULATORY PROTEIN (PROB...	27	1.00000	2
Mb0564c CONSERVED HYPOTHETICAL PROTEIN [FIRST PART] 64157...	28	1.00000	1
Mb2901 POSSIBLE CONSERVED TRANSMEMBRANE PROTEIN 3144179:3...	28	1.00000	1
Mb2505c CONSERVED HYPOTHETICAL PROTEIN 2752283:2752768 re...	24	1.00000	3
Mb1026 HYPOTHETICAL PROTEIN 1116218:1116976 forward MW:25969	23	1.00000	3
Mb0833c CONSERVED HYPOTHETICAL PROTEIN 905735:905917 reve...	25	1.00000	1
nuoM PROBABLE NADH DEHYDROGENASE I (CHAIN M) NUOK (NADH-U...	36	1.00000	1
Mb2672c PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN 2932...	20	1.00000	2
Mb1251c PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN 13639...	26	1.00000	3
fdxA PROBABLE FERREDOXIN FDXA 2235000:2235344 reverse MW:...	22	1.00000	2
Mb1436 POSSIBLE MEMBRANE PROTEIN 1573237:1573839 forward ...	31	1.00000	1
uspA PROBABLE SUGAR-TRANSPORT INTEGRAL MEMBRANE PROTEIN A...	24	1.00000	2
Mb2308c conserved hypothetical protein [FIRST PART] 25367...	26	1.00000	2
Mb3773 CONSERVED HYPOTHETICAL PROTEIN 4133018:4133401 for...	22	1.00000	2
echA11 PROBABLE ENOYL-COA HYDRATASE ECHA11 (ENOYL HYDRASE...	26	1.00000	2
bpoA POSSIBLE PEROXIDASE BPOA (NON-HAEM PEROXIDASE) 38376...	32	1.00000	1
Mb2332c HYPOTHETICAL GLYCINE RICH PROTEIN 2557099:2557530...	22	1.00000	2
Mb0634 CONSERVED HYPOTHETICAL PROTEIN 712251:712652 forwa...	25	1.00000	2
trxB2 PROBABLE THIOREDOXIN REDUCTASE TRXB2 (TRXR) (TR) 43...	33	1.00000	1
Mb0084 PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN 89610:...	16	1.00000	3
ogt PROBABLE METHYLATED-DNA--PROTEIN-CYSTEINE METHYLTRANS...	18	1.00000	3
Mb0512 CONSERVED HYPOTHETICAL PROTEIN 592262:592498 forwa...	19	1.00000	2
Mb2123c CONSERVED HYPOTHETICAL PROTEIN 2336155:2337153 re...	31	1.00000	2
Mb0112c HYPOTHETICAL PROTEIN 130932:131141 reverse MW:7590	24	1.00000	1
Mb2778c CONSERVED HYPOTHETICAL PROTEIN 3026744:3027160 re...	20	1.00000	2
Mb0901 CONSERVED HYPOTHETICAL PROTEIN 976541:977329 forwa...	23	1.00000	2
Mb1663c CONSERVED HYPOTHETICAL PROTEIN 1828264:1829058 re...	21	1.00000	3
Mb0644c CONSERVED HYPOTHETICAL PROTEIN 720007:721158 reve...	33	1.00000	1
thiD PROBABLE PHOSPHOMETHYLPYRIMIDINE KINASE THID (HMP-PH...	23	1.00000	3
rubA PROBABLE RUBREDOXIN RUBA 3585171:3585338 reverse MW:...	17	1.00000	2
Mb2627 CONSERVED HYPOTHETICAL PROTEIN 2893191:2893595 for...	25	1.00000	2
Mb0655 CONSERVED HYPOTHETICAL PROTEIN 733629:734057 forwa...	21	1.00000	2

Mb3404 CONSERVED HYPOTHETICAL PROTEIN 3734618:3735052 for... 21 1.00000 2
lppOb Probable conserved lipoprotein lppOb 2540344:254070... 16 1.00000 3
Mb1319 CONSERVED HYPOTHETICAL PROTEIN 1439494:1440711 for... 33 1.00000 1
Mb2322c CONSERVED HYPOTHETICAL PROTEIN 2549671:2550603 re... 27 1.00000 2
Mb2634 CONSERVED HYPOTHETICAL PROTEIN 2897801:2898241 for... 21 1.00000 2
PPE57 PPE FAMILY PROTEIN 3794867:3795403 forward MW:19799 21 1.00000 3
Mb3887c POSSIBLE MEMBRANE PROTEIN 4265690:4265887 reverse... 23 1.00000 1
Mb2995 CONSERVED HYPOTHETICAL PROTEIN 3282623:3282793 for... 23 1.00000 1
Mb0399 CONSERVED 13E12 REPEAT FAMILY PROTEIN 473800:47512... 33 1.00000 1
Mb2448c PROBABLE TRANSPOSASE [FIRST PART] 2689667:2689960... 22 1.00000 2
cspA PROBABLE COLD SHOCK PROTEIN A CSPA 4025565:4025768 r... 23 1.00000 1
Mb2465 CONSERVED HYPOTHETICAL PROTEIN 2704872:2705150 for... 25 1.00000 1
ruvC PROBABLE CROSSOVER JUNCTION ENDODEOXYRIBONUCLEASE RU... 22 1.00000 3
Mb2350c CONSERVED HYPOTHETICAL PROTEIN 2572956:2573864 re... 26 1.00000 2
Mb0943 CONSERVED HYPOTHETICAL PROTEIN 1025150:1025650 for... 28 1.00000 2
Mb2738c POSSIBLE CONSERVED MEMBRANE PROTEIN 2987676:29881... 28 1.00000 1
Mb0255c PUTATIVE SUCCINATE DEHYDROGENASE [MEMBRANE ANCHOR... 25 1.00000 2
moaB2 POSSIBLE PTERIN-4-ALPHA-CARBINOLAMINE DEHYDRATASE M... 24 1.00000 2
esxL PUTATIVE ESAT-6 LIKE PROTEIN 4 1342253:1342537 forwa... 16 1.00000 3
Mb3634c PROBABLE CONSERVED TRANSMEMBRANE PROTEIN RICH IN ... 32 1.00000 1
Mb3768c POSSIBLE OXIDOREDUCTASE 4129144:4129539 reverse M... 25 1.00000 2
esxT CONSERVED HYPOTHETICAL PROTEIN 3810325:3810627 rever... 25 1.00000 1
Mb1599 Probable phiRV1 phage protein 1764680:1765090 forw... 19 1.00000 3
Mb0719 HYPOTHETICAL PROTEIN 801397:801618 forward MW:7391 16 1.00000 2
Mb1004c HYPOTHETICAL PROTEIN 1095121:1095315 reverse MW:7127 17 1.00000 2
Mb0082 HYPOTHETICAL PROTEIN 88239:89060 forward MW:29507 30 1.00000 1
Mb2758c CONSERVED HYPOTHETICAL PROTEIN 3008442:3008648 re... 16 1.00000 2
lprB PUTATIVE LIPOPROTEIN LPRB 1422343:1422900 forward MW... 20 1.00000 3
Mb3393 CONSERVED HYPOTHETICAL PROTEIN 3724571:3724828 for... 18 1.00000 2
Mb0031 HYPOTHETICAL PROTEIN 33208:33537 forward MW:11858 14 1.00000 3
Mb1945c PROBABLE EXPORTED PROTEIN 2146986:2147579 reverse... 26 1.00000 2
echA10 PROBABLE ENOYL-CoA HYDRATASE ECHA10 (ENOYL HYDRASE... 23 1.00000 2
Mb1559c CONSERVED HYPOTHETICAL PROTEIN 1717129:1717563 re... 22 1.00000 2
gmhA PROBABLE PHOSPHOHEPTOSE ISOMERASE GMHA 137357:137947... 28 1.00000 1
Mb2556 CONSERVED HYPOTHETICAL PROTEIN 2818112:2818513 for... 26 1.00000 1
Mb2600 CONSERVED HYPOTHETICAL PROTEIN 2861961:2862350 for... 20 1.00000 2
bphC PUTATIVE BIPHENYL-2,3-DIOL 1,2-DIOXYGENASE BPHC (23O... 30 1.00000 1
Mb0581c CONSERVED HYPOTHETICAL PROTEIN 658791:659282 reve... 17 1.00000
tesB2 PROBABLE ACYL-COA THIOESTERASE II TESB2 (TEII) 2899... 28 1.00000 2
Mb1994c CONSERVED HYPOTHETICAL PROTEIN 2195315:2195611 re... 19 1.00000 2
Mb3501 CONSERVED HYPOTHETICAL PROTEIN 3837058:3837564 for... 27 1.00000 1
Mb2542 HYPOTHETICAL PROTEIN 2796958:2797380 forward MW:14956 26 1.00000 1
Mb0990c CONSERVED HYPOTHETICAL PROTEIN 1077217:1077636 re... 24 1.00000 2
Mb0626 CONSERVED HYPOTHETICAL PROTEIN 705075:705302 forwa... 17 1.00000 2
Mb2279c HYPOTHETICAL PROTEIN 2508042:2508236 reverse MW:6972 17 1.00000 2
TB27.3 CONSERVED HYPOTHETICAL PROTEIN TB27.3 672409:67319... 29 1.00000 1
Mb0619 POSSIBLE EXPORTED PROTEIN 701101:701412 forward MW... 23 1.00000 2
Mb1965c CONSERVED HYPOTHETICAL PROTEIN 2173553:2174077 re... 27 1.00000 1

Table 5: Sixty six genes coding for proteases in *M. tuberculosis* genome

Number	ORF	Gene Name	Function	Mass	PI	Secretion signal		Zinc-binding motif (PROSITE PDOS00129)	Comments about function	Sequence similarity test
						ExProt	SignalP			
1	Rv3305c	AmiA1	Unknown	41226	5.82	_	_		Involved in cellular metabolism. It hydrolyses L-amino acid and amides	N/A
2	Rv3306c	AmiB1	Active on carbon aliphatic amides and/or many aromatic amides	40740	4.85	_	_			N/A
3	Rv0734	MapA	Remove the amino-terminal methionine from nascent protein	27276	5.54	_	_			N/A
4	Rv2861c	MapB		30890	4.87	_	_			
5	Rv3330	DacB1	Peptidoglycan synthesis	41682	6.23	+	+		Involved in peptidoglycan synthesis (at final stages). It hydrolyzes the bound D-alanyl-D-alanine	N/A
6	Rv2911	DacB2		29746	4.81	+	+			
7	Rv3610c	FtsH	Cell division protein	81985	5.60	_	_	Yes	Thought to act as an ATP-Dependent zinc metallopeptidase.Probably it has a regulatory role in stress response and specific protein secretion for adaption to host environment	N/A
8	Rv3419c	Gcp	Hydrolysis O-sialoglycoproteins	35090	5.34	_	_		GcP is probable a O-sailoglycoprotein endopeptidase	N/A

9	Rv0198c	Zmp1	Unknown	73881	4.84	_	_	Yes	(i) Essential for prevention of inflammasome activation and IL-1β production, (ii) required for mycobacterial survival in macrophages, (iii) necessary for full virulence in a murine model of tuberculosis (Master et al., 2008).	N/A
10 11 12 13 14	Rv0384c Rv3596c Rv2457c Rv2460c Rv2461c	ClpB ClpC ClpX ClpP2 ClpP1	Degradation of misfolded proteins	92536 93552 46782 23507 21675	4.97 5.65 4.78 4.75 4.54	_ _ _ _ _	_ _ _ _ _		Clp family cleaves peptide in various proteins in a process that requires ATP hydrolysis. It plays a major role in the degradation of misfolded proteins. It shows a chymotrypsin-like activity. ClpB – thought to be ATPase subunit of an intracellular ATP-dependent protease. It seems to be regulated positively by sigH (Rv3223c product) and negatively by hsp R (Rv0353 product)	
15	Rv2725c	Hflx	Possibly a putative GTPase, modulating activity of HflK and HflC	53327	6.80	_	_			N/A
16	Rv1223	HtrA3	Degradation of unfolded protein	54157	4.73	+	_		HtrA is a family of heat-shock proteins that is required for growth elevated temperature	
17	Rv0983	PepD	Unknown	46452	6.29	+	_			N/A
18 19	Rv2213 Rv0800	PepB PepC	Protein degradation	53449 46012	8.21 6.54	+ _	_ _		PepB, PepC and PepN are probable amino peptidase. They hydrolyze peptides or proteins. PepE and PepQ are probable peptidases. PepR is probable zinc protease	

20	Rv2089	PepE	hydrolysis of peptide bonds	39439	4.48	_	_	Yes		N/A
21	Rv2467	PepN	Probable amino peptidase	94252	4.68	_	_			N/A
22	Rv2535	PepQ	Probable peptidase	38758	5.12	_	_	Yes		N/A
23	Rv2782c	PepR	Zinc protease	47072	7.50	_	_			N/A
24	Rv2109c	PrcA	Protein degradation	26848	5.18	_	_		Proteasome subunits	
25	Rv2110c	PrcB	Protein degradation	30272	4.41	_	_			N/A
26	Rv0781	PtrB	Cleaves peptide bonds	26915	5.95	_	_		Cleaves peptide bonds on C-terminal side of lysyl and argininyl residues	
27	Rv0724	SppA	Involved in digestion of the cleaved signal peptide	65935	5.82	_	_			N/A
28	Rv3915	Rv3915	Unknown	43911	6.84	_	_		This activity is necessary to maintain proper secretion of mature proteins across the membrane	
29	Rv2141c	Rv2141c	Unknown	48110	4.71	_	_			N/A
30	Rv0457c	Rv0457c	Unknown	74463	4.48	_	_			N/A
31	Rv3100c	SmpB	Binds specifically to the SSRA RNA (TMRNA)	18197	11.33	_	_		It is required for stable association of SSRA with ribosomes. Thought to be implicated in the survival of bacterium within macrophage	
32	Rv3671c	Rv3671c	Unknown	40721	6.79	_	_		Probable hydrolyses peptides and/or proteins (possibly cleaved preferentially after serine residues)	
33	Rv2869c	Rip	Controls membrane composition	42834	8.69	_	_			N/A

34	Rv0563	HtpX	Probable heat shock protein	30682	10.20	_	_	Yes	Possibly involved in adaption. It hydrolyzes specific peptides and/or proteins	N/A
35	Rv0840c	Pip	Release of a N-terminal proline from a peptide	31893	5.36	_	_			N/A
36	Rv1977	Rv1977	Unknown	38307	7.68	_	_	Yes		N/A
37	Rv0319	Pcp	Removes 5-Oxoproline from various penultimate amino acid residues except L-proline	23192	6.50	_	_			N/A
38	Rv2903c	LepB	It cleaves the N-terminal leader sequences from secreted protein precursors	31848	5.72	+	_		Probable a signal peptidase I (space I or leader peptidase I)	N/A
39	Rv1539	LspA	Catalyzes the removal of signal peptides from prolipoprotein	21312	6.53	_	_		Probable a signal peptidase II	N/A
40	Rv2224c	Rv2224c	Unknown	55892	6.07	+	_			N/A
41	Rv0419	LpqM	Unknown lipoprotein	52948	5.05	+	_	Yes		N/A
42	Rv0470c	UmaA2	Mycolic acid synthase	33027	6.44	_	_	Yes		N/A
43	Rv2017	Rv2017	Probable regulator	38605	7.14	_	_	Yes		N/A
44	Rv2189c	Rv2189c	Unknown	27093	5.55	_	_	Yes		N/A
45	Rv2515c	Rv2515c	Unknown	45767	6.10	_	_	Yes		N/A
46	Rv2711	IdeR	Iron-dependent suppressor	25232	5.04	_	_	Yes		N/A
47	Rv3430c	Rv3430c	Possible transposase	43317	10.01	+	_	Yes		N/A

48	Rv3365c	Rv3365c	Unknown	93409	6.92	_	_	Yes		N/A
49	Rv3626c	Rv3626c	Unknown	38406	7.32	_	_	Yes		N/A
50	Rv3883c	MycP1	Probable membrane-anchored proteins	45085	4.98	+	+		A family of subtilisin-like serine proteases. The mycosin have N-terminal signal sequences and C-terminal trans membrane anchors. All mycosins are expressed constitutively during growth in broth. (Brown GD et.al, 2000). Proteolyzed EspB in periplasm. In active MycP1 protease mutant causes hyper activation of ESX-1 stimulated innate signaling pathways during macrophage infection. MycP1 is required for virulence in macrophages and mice that identified by quantitative PCR , western blot and immunoblot for supernatant of *M. tuberculosis.* (Yamini M. Ohol et.al, 2010)	N/A
51	Rv3886c	MycP2		55594	5.90	+	+			
52	Rv0291	MycP3		46127	5.14	+	+			
53	Rv3449	MycP4		46015	6.62	+	+			
54	Rv1796	MycP5		60027	5.43	+	+			
55	Rv2672	Rv2672	Unknown	54015	4.72	+	+	Yes		N/A
56	Rv3435c	Rv3435c	Unknown	30604	8.46	+	+		Identified as outer membrane protein by western blot and ELISA. (Song et.al, 2008)	N/A
57	Rv0185	Rv0185	Unknown	18364	7.32	_	+	Yes		N/A
58	Rv0359	Rv0359	Unknown	28089	9.04	+	+	Yes		N/A

59	Rv2515	Rv2515	Unknown	45767	6.10	_	+			N/A
60	Rv2223c	Rv2223c	Unknown	55077	4.70	+	+	Yes	Identified as a cell wall protein of *M. tuberculosis* by a combination of detergent extraction, 2DGE, multidimensional liquid chromatography, and mass spectrometry. (Lisa M. Wolfe et.al,2010)	N/A
61	Rv2625c	Rv2625c	Unknown	41480	10.22	+	_	Yes	*M. tuberculosis* H37Rv Integral Membrane Proteins identified by One-Dimensional Gel Electrophoresis and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. (Ying Xiong et.al, 2005)	N/A
62	Rv3207c	Rv3207c	Unknown	31033	6.11	+	+	Yes	Conserved hypothetical protein. It was identified by mass spectrometry in the culture filtrate of M. tuberculosis H37Rv but not the membrane protein fraction or whole cell lysates (de Souza et al., 2011).	N/A
63	Rv3836	Rv3836	Unknown	14994	3.90	_	+	Yes		N/A
64	Rv2575	Rv2575	Unknown	30806	4.74	+	+	Yes		Found in *Brucella.melitensis* As neutral zinc metallopeptidase
65	Rv0125	PepA	Unknown	34926	4.90	+	+		Identified in culture filtrate protein of *M. tuberculosis* H37Rv by 2-DE combined with MALDI-TOF MS and LC coupled MS/MS. (Hiwa Malen et.al,2007)	Found the similar gene PepA (HtrA) in*A.pleuropneumoniae* . HtrA is a peripheral membrane protein,lying on

										the periplasmic side of inner membrane (Mark J.Pallen and Brendan W. Wren,1997) Found in *Bacillus.thuringiensis* . It is metalloprotease produced during the early stages of sporulation (Li.E and Yousten.A,1975)
66	Rv3668c	Rv3668c	Unknown	23102	5.07	+	+			Found in *C. diphtheria* as putative protease. Also, found in *Myxococcus.fulvus* as family peptidase. Found in*N.farcinica* as putative protease. Additionally, found in *R..equi* as putative secreted peptidase. Found in *S.coelicolor* as secreted serine protease.

N-terminal sequencing result for isolated protease

Mazen Saleh
Laurentian University
Biology

SEQUENCING ANALYSIS SUMMARY

Customer Code	Sample Name	Sample Code	Analysis Date
SalehM	Louai PVDF	033015CA	2015-03-30

Residue:	Amino Acid:	Comments:
Residue 1		Possible amino acids for Residue 1: S-Ser; G-Gly; V-Val
Residue 2	T - Thr	
Residue 3		Possible amino acids for Residue 3: G-Gly
Residue 4		Possible amino acids for Residue 4: G-Gly
Residue 5		Possible amino acids for Residue 5: G-Gly
Residue 6	A - Ala	
Residue 7		
Residue 8		
Residue 9		
Residue 10		

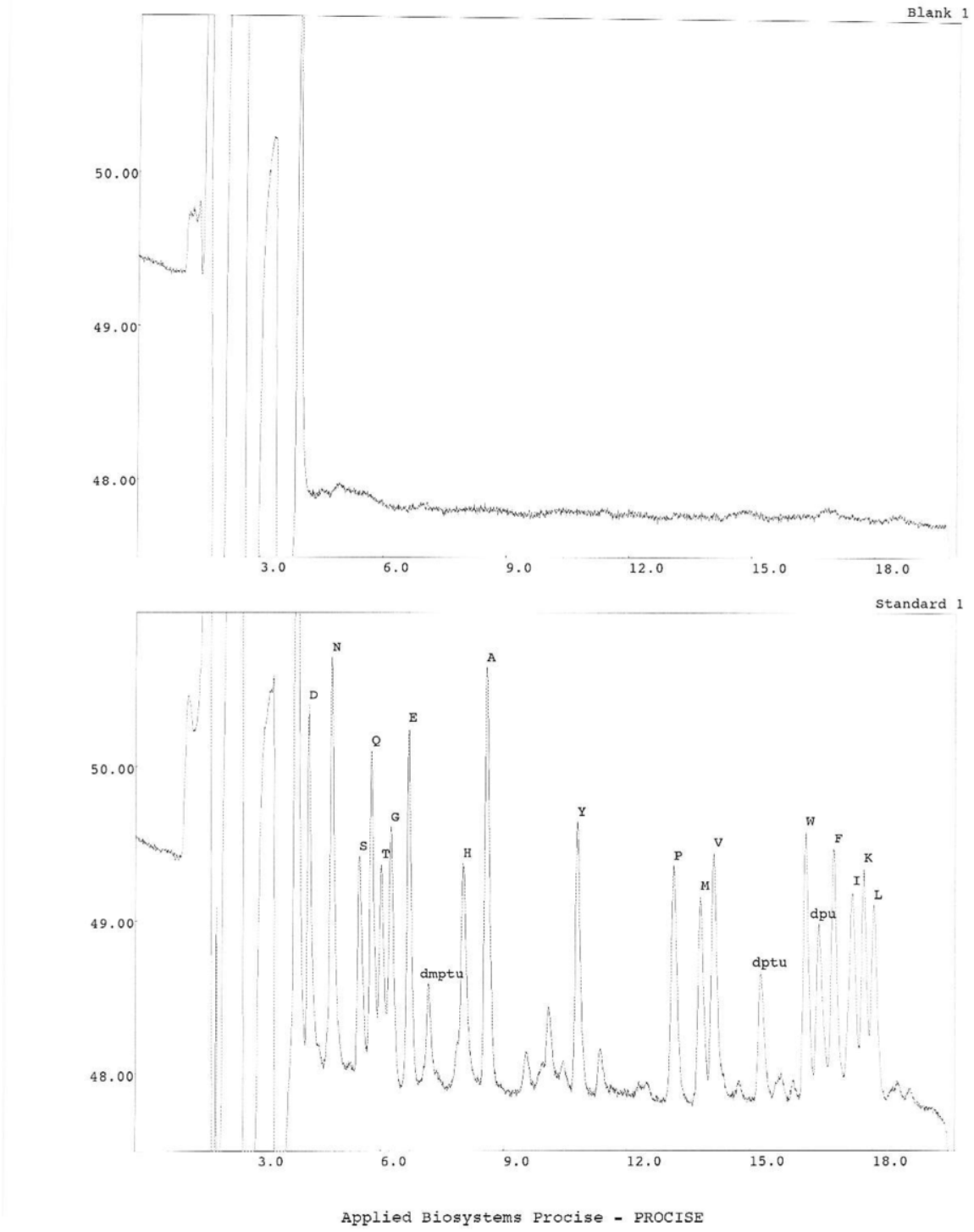
File Name: 033015CA
In Folder: Macintosh HD:Proisef:Mar 2015:
8 cycles acquired on an Applied Biosystems Procise Sequencer
Sequencer Name: PROCISE
Run started on Monday, March 30, 2015 at 3:03 PM

Sample Name:	033015CA		
Sample Amount:	1.0 picomoles	Sampling Rate:	4.0 Hertz
Standard Amount:	10.0 picomoles	Detector Scale:	1.000 AUFS

Analyzed Using Smooth Degree 9, Interpolated Baseline

Analysis Limits:	1 - 128
Integration Limits:	3.50 - 19.00 min.
Baseline Limits:	0.10 - 1.00 min.
Peak Half Width:	0.05 min.
Peak Separation:	0.05 min.
Search Length:	0.10 min.
RT Factor:	0.75
Sensitivity:	1.00

Applied Biosystems Procise - PROCISE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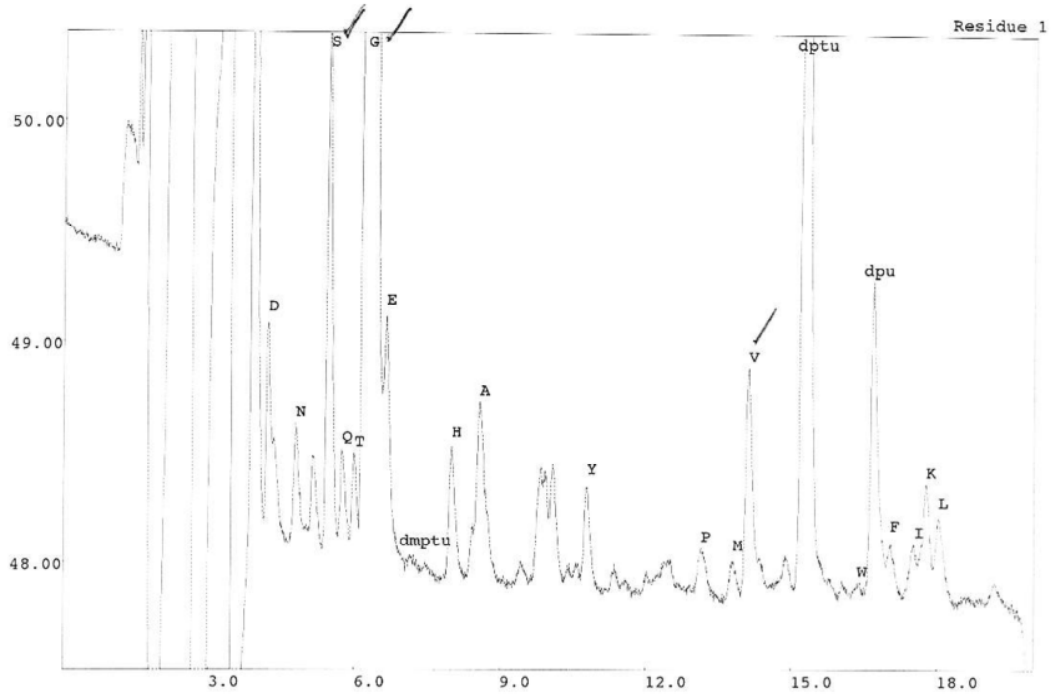

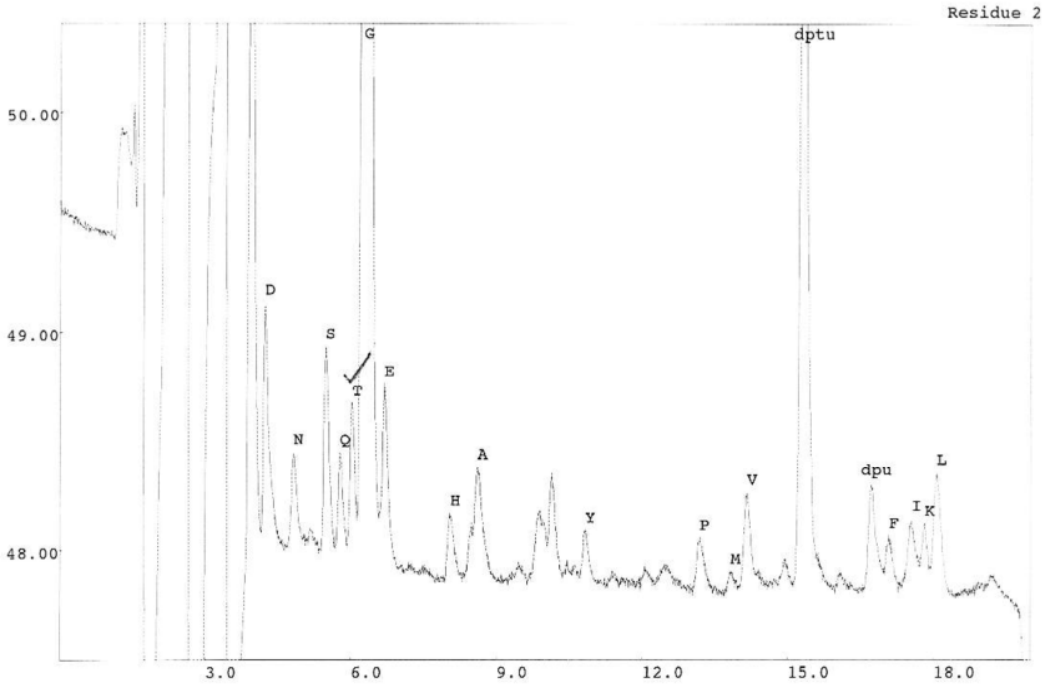

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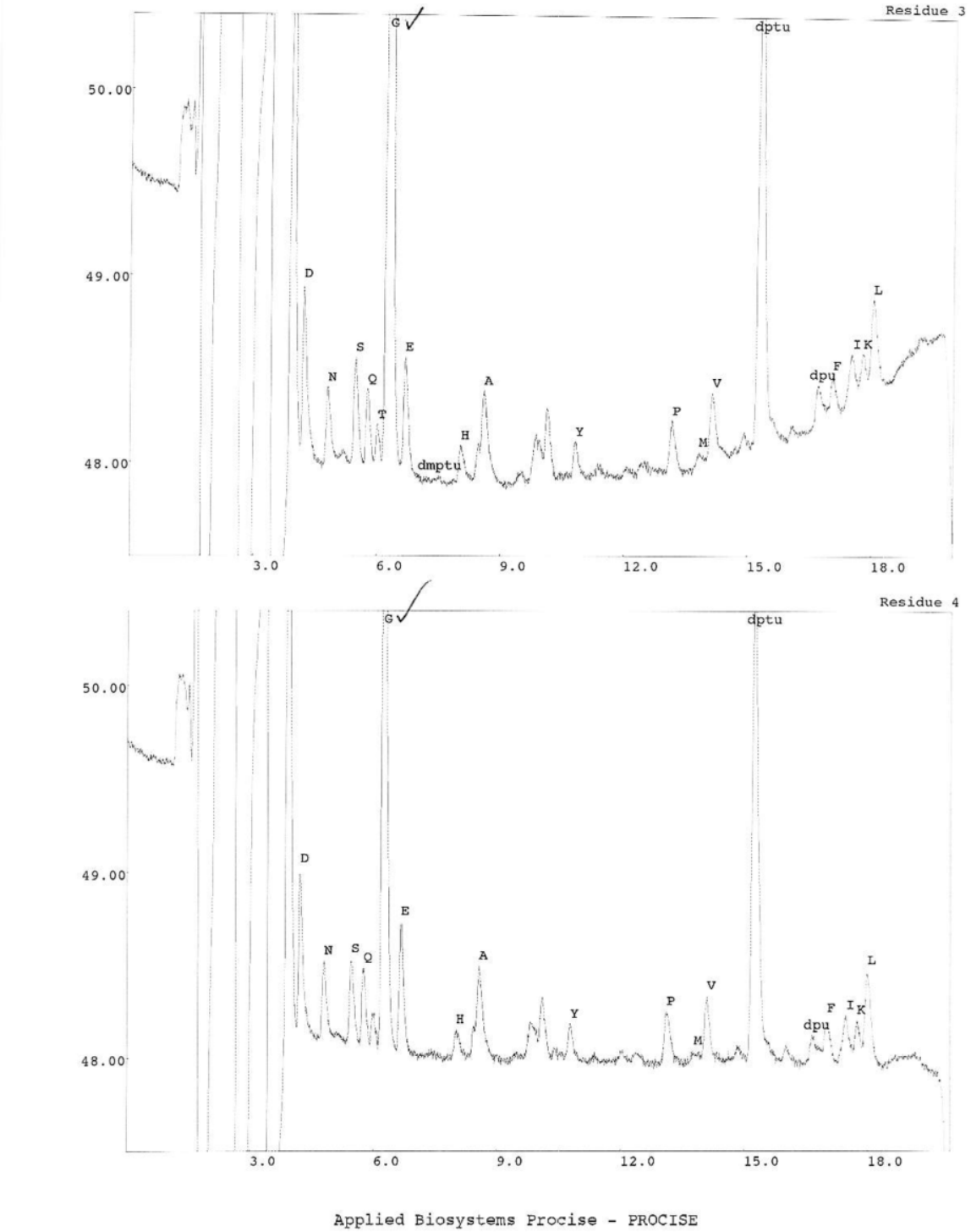

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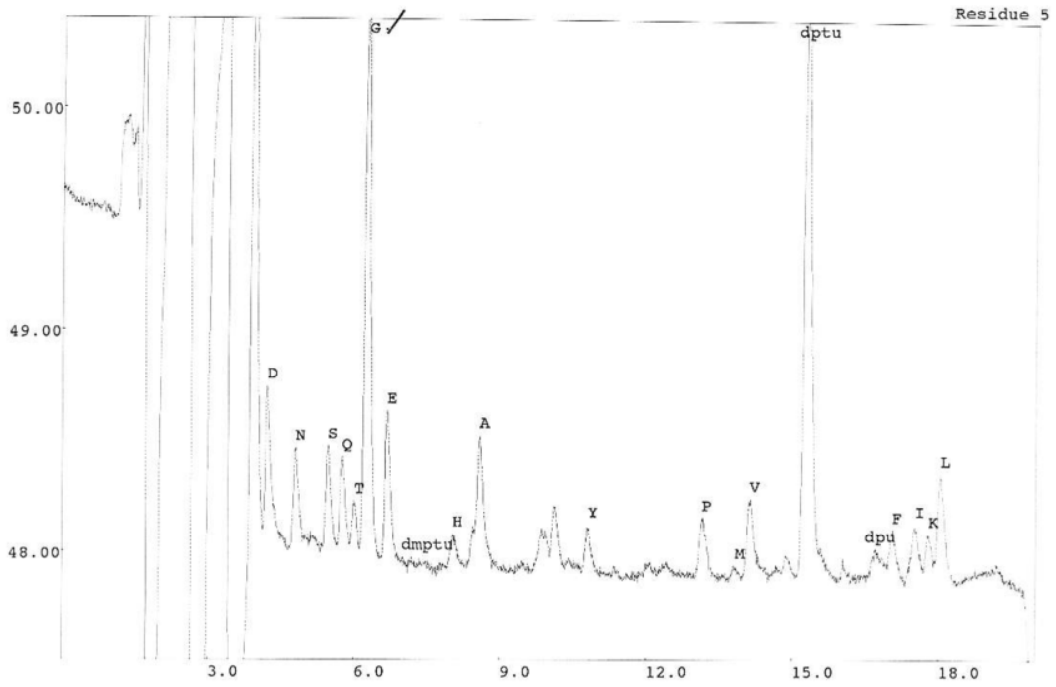

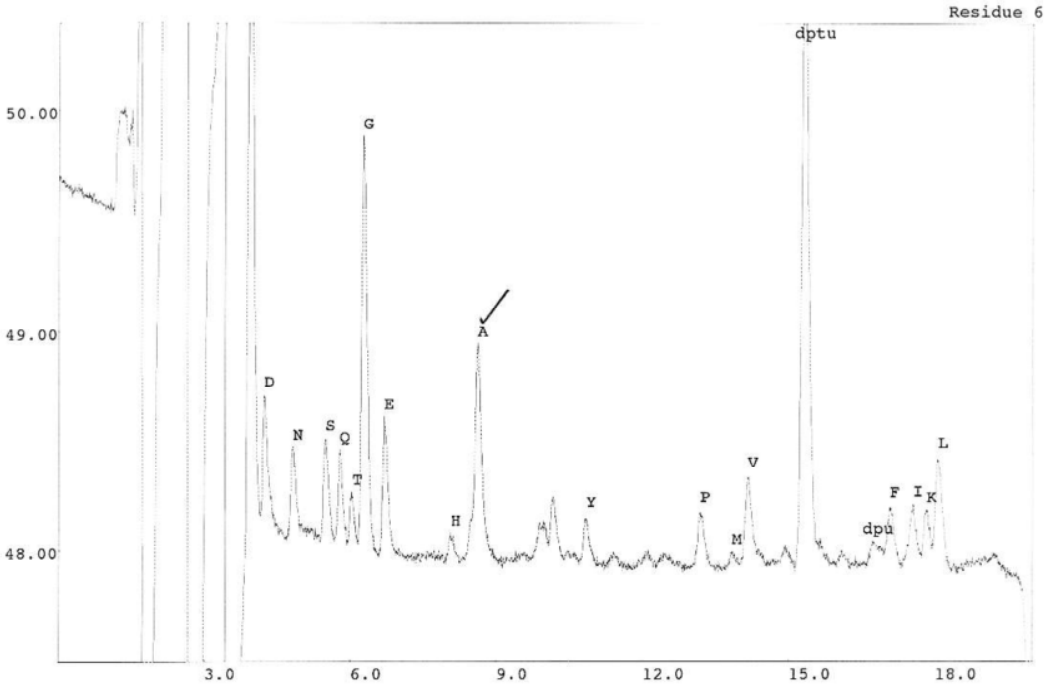

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