

**EXPRESSION AND CHARACTERIZATION OF
MYCOBACTERIUM PARATUBERCULOSIS 19KDA
WITH POSTTRANSLATIONAL MODIFICATION**

by

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ABSTRACT

Despite the fact that *E. coli* supports limited posttranslational modification, this bacterium has been universally used as the expression system of choice. Expression of modified proteins in *E. coli* may lead to expression of recombinant proteins that lack essential immunomodulatory or catalytic components essential for infectious processes. Previously in our laboratory, pMptb#28 plasmid containing a 4.8 kb insert from *M. paratuberculosis* has been identified which expressed 16 kDa recombinant protein in *E. coli* and 19 kDa recombinant protein in *Mycobacterium smegmatis*. The objective of this study is to identify the ORF sequence, investigate possible posttranslational modification and characterize the protein forms in the two hosts. Earlier in the study, the genome sequence for *M. paratuberculosis* was not available and therefore sequencing both the 5' and 3' ends of the 4.8 kb insert did not help in the identification of the ORF. However, unidirectional Exonuclease deletion resulted in identification of subclones containing possible ORF sequence. Later on, the publication of the *M. paratuberculosis* genome sequence along with BLAST analysis of sequences from the subclones resulted in the identification of 486 bp ORF with significant identity to that from *M. tuberculosis* and *M. leprae*. Cloning of the 486 ORF coding sequence in *E. coli* resulted in the expression of 16 kDa protein similar to the calculated predicted size of translated peptide. Cloning of the 486 bp ORF coding sequence in *M. smegmatis* resulted in the expression of 19 kDa protein similar to that from *M. paratuberculosis*. The 16/19 kDa forms of the same protein were verified using rabbit anti-*M. paratuberculosis* antibodies adsorbed in *E. coli* and *M. smegmatis* lysates. The size of the 19 kDa proteins was not reduced following

treatment with deglycosylation enzymes in absence of any enzyme inhibitors. The 19 kDa product was confirmed not be a glycoprotein when failed to react with ConA stain. The 16/19 kDa forms of the protein were evaluated against T-lymphocytes from Crohn's disease patients and normal controls. T- proliferation assay included controls such as PHA and PPD from *M. paratuberculosis*. There was not a significant difference between the two forms of the protein (16/19 kDa) against T-cell response from both populations. Overall, the study identified the ORF of the 19 kDa non-glycoprotein from *M. paratuberculosis*. Moreover, this is the first study which reports that the zoonotic *M. paratuberculosis* supports posttranslational modification similar to *M. tuberculosis* and *M. leprae* pathogens. Although the posttranslational modification component in this 19 kDa nonglycoprotein did not affect T- cell response, the finding is significant toward glycoproteins from *M. paratuberculosis* and their role in the pathogenesis of this bacterial infection in animals and humans.

I would like to dedicate my work to my husband Amir for his endless support, encouragement, patience and love. For taking care of everything so I would not have to worry about anything but my work here at UCF.

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LIST OF ABBREVIATIONS

ATCC: American Type Culture Collectio

BrdU: Bromodeoxyuridine

CD: Crohn's Disease

EDTA: Ethylenediaminetetraacetic acid

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

OADC:Oleic acid-Albumin-Dextrose-Catalase

PBMC: Peripheral blood mononuclear cells

PHA: Phytohemagglutinin

PPD: purified protein derivative

PWM: Pokeweed mitogen

RPMI-1640: Roswell Park Memorial Institute

WHO: World Health Organization

CHAPTER ONE

INTRODUCTION

Most members of the genus *Mycobacterium* are important pathogens of humans and animals. In humans, leprosy and tuberculosis (TB), caused by *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively, continue to be serious public health problems and leading causes of death world-wide. The incidence of leprosy is 15 million world-wide, with approximately 30% of its victims suffering severe disfiguration (37, 21).

Tuberculosis is a highly contagious disease with over 8 million new cases and 3 million fatalities occurring annually as estimated by World Health Organization (WHO) (1, 4, 11, 31). The synergy between HIV and TB, combined with the emergence of multidrug-resistant strains of *M. tuberculosis* in several parts of the world has fueled fears of the spread of TB in the near future (1,2) *M. avium* complex (MAC) causes disseminated diseases especially in persons with AIDS (60). Nair et al. (1992) reported that 50% of individuals with AIDS develop MAC disease symptoms. *M. paratuberculosis*, the causative agent of Johne's disease, causes enormous economic loss in the dairy industry (8, 62, 28). In the United States, prevalence studied has estimated that between 20 to 30% of dairy herds are infected with *M. avium subsp. paratuberculosis* as a result over \$200 million in lost annual revenue to the dairy industry (10, 28, 61).

M. paratuberculosis has been proposed to be associated with Crohn's disease, a painful debilitating chronic inflammatory bowel disease of the GI tract following the identification of this bacterium in intestinal tissue from Crohn's disease patients (40, 52).

The association has been vigorously debated in recent years especially following the

consistent isolation of *M. paratuberculosis* from tissue, milk and blood from humans with Crohn's disease (53, 42, 9, 43, 54).

Although drug therapy can be effective against some mycobacterial infections, vaccines offer the best hope of controlling these diseases and eliminating their agents.

Development of vaccines and improved therapies and diagnostic methods will require a more basic understanding of mycobacterial antigens and their encoding genes. Small quantities of immunogenic proteins identified in mycobacterial cultures, slow growth of pathogenic mycobacteria (*M. tuberculosis*, *M. paratuberculosis*) or not at all (*M. leprae*) in laboratory, and safety precautions needed to work with pathogenic bacteria are some of the barriers that make difficulties in evaluating of these antigens (27). The development of recombinant protein technology to express important genes in *E. coli* has been well established. However, limited success has been reported following attempts to express some mycobacterial genes in *E. coli* cloning expression system. The variability in the %G+C content and the nature of the mycobacterial cell wall between *E. coli* and *Mycobacterium* may contribute significantly to the variation. Additionally, recent studies have shown some post-translational modifications in mycobacterial antigens such as acylation and glycosylation (20, 25), which is lacking in the majority of Gram negative bacteria such as *E. coli*. Consequently, expression of some mycobacterial proteins in *E. coli* expression system, may lead to expression of limited quality and nature of cloned genes. There is great need for cloning mycobacterial genes and expression of encoding antigens in a homologous host which may provide as an alternative for expression of proteins similar to those from the native host (59, 27). Therefore, performing these manipulations in a homologous host has the advantage of permitting possible

posttranslational pathways that may include immunomodulatory components. For example, it has been found that functionally active superoxide dismutase was expressed by mycobacterial system, in contrast to the enzymatically inactive protein produce in *E. coli* (63). Another example showed decreasing capacity of *E. coli* recombinant 45/47-kDa molecules (Apa) of *M. tuberculosis* to stimulate T lymphocyte responses (27).

The first attempt to achieve that was initiated by constructing a chimeric shuttle plasmid containing a mycobacteriophage DNA inserted into an *E. coli* cosmid (29). This shuttle plasmid replicated in *E. coli* as a plasmid and in mycobacteria as phages. This was followed by construction of many shuttle plasmids derived from the *M. fortuitum* plasmid pAL5000 (33). Most of these shuttle plasmids were characterized by their large size and lacked sufficient genetic elements. Consequently, the introduction and expression of foreign genes and their encoding products in mycobacteria requires use of efficient shuttle plasmids.

M. paratuberculosis continues to emerge as significant pathogen to humans with Crohn's disease and animals with Johne's disease. Understanding virulence mechanism in this bacterium will aid in understanding its pathogenesis and ultimately controlling it. In this study, we attempt to investigate *M. paratuberculosis* for its ability to support posttranslational modification. Ultimately more questions need to be answered concerning the role of such modification in this important pathogen. In this laboratory, a previously constructed genomic library of *M. paratuberculosis* in *E. coli* revealed the identification of the Clone pMptb #28 containing 4.8 kb insert and the expression of a single 16 kDa protein product. When the 4.8 kb was cloned in *M. smegmatis* using pNEZ6.3 shuttle plasmid, the expressed product was estimated at 19 kDa similar to the

size present in *M. paratuberculosis*. The main objective in this study is to identify the ORF nucleotide sequence of the encoding gene followed by cloning it in *E. coli* and *M. smegmatis*. The outcome of this study will determine whether *M. paratuberculosis* supports posttranslational modification or not. If it does this, the nature of the posttranslational modification would be investigated for possible glycosylation and its role in the antigenicity of the protein against T-lymphocyte derived from Crohn's disease patients and normal controls.

CHAPTER TWO

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli strain TOP10 purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA) and its recombinants were grown in Luria Britani (LB) broth media (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v NaCl) supplemented with ampicillin and/or kanamycin (Sigma) at a final concentration of 50ug/ml. The 2 ml starting cultures were incubated at 37 °C with agitation at 175 rpm for 16 hours until OD₆₀₀ achieves 1.2 to 1.5. The overnight culture was further transferred and grown in the large volume of LB broth in the similar condition. The *E. coli* clone containing the plasmid pMptb#28 was obtained from our genomic library as described previously (15).

M. smegmatis mc2155, an efficient plasmid transformation mutant of *M. smegmatis* strain ATCC 607 isolated by Snapper *et al.* (1988), was obtained from Dr. Charlotte McCarthy (Department of Biology, New Mexico State University, Las Cruces, NM). *M. paratuberculosis* strain linda (ATCC 43015), a clinical strain isolated from a Crohn's disease patient by Chiodini *et al.* (1984) was obtained from Dr. P. Brennan (Department of Microbiology, Colorado State University, Fort Collins, CO). They were grown in Middlebrook 7H9 broth supplemented with OADC enrichment (Difco Laboratories, Detroit, MI), 0.5% glycerol and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo). Mycobactin J (Allied Monitor Inc., Fayette, Mo) was added to the broth for culturing *M.*

paratuberculosis at 2 mg/L as described by Graham *et al.* (1987). The inoculated broth was incubated at 37 °C on a rotary shaker (150 rpm) until an optical density OD₆₀₀ of 1.8 (48 hours for *M. smegmatis* and 4-6 weeks for *M. paratuberculosis*). *M. smegmatis* kanamycin resistant recombinants were isolated on 7H10 agar plates supplemented with OADC, glycerol (0.5%v/v) and 50 ug/ml kanamycin. An *E. coli* clone containing the shuttle plasmid pAL32 (Labidi *et al.*, 1985) was obtained from Labidi (Cytogenetics Vaccine Co., Dallas, TX).

Shuttle Plasmid Construction

The shuttle plasmid pNEZ6.3 used in this study was previously constructed by Naser *et al.* (1996). The *ori* of pBR322 is located upstream of Sp6 promoter whereas the *f1 ori* is located upstream of the T7 promoter. In addition, it contains the *ori* of pAL5000 and the Kan resistant gene(Km). The shuttle plasmid Pmip12 (6800bp), was also used in this experiment (36). It carries the pAL5000 origin of replication, promoter derived from *mycobacterium fortuitum*, and Kan resistance gene (36).

Plasmid DNA Extraction

Plasmid DNA from *M. smegmatis* and kanamycin resistant recombinants was prepared following a modification of that described by Kado and Liu, 1981 (32). Briefly, the cell pellet from 30 ml of culture broth was suspended in 2.0 ml TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), mixed with lysozyme (50 mg/ml) and then incubated at 37°C on a rotary shaker (100 rpm) for 18 hours. Four ml of lysing buffer (3% SDS, 50 mM Tris-Cl, pH 11.7) was added, mixed and then incubated at 68°C for 30 min. The lysate was extracted with phenol:isoamyl alcohol chloroform (1:1, v/v) and then precipitated with ethanol. Plasmid DNA from *E. coli* was prepared by Qiagen (QIAGEN Inc., Chatsworth, CA) as

described by the manufacturer. The extracted plasmid DNA was further digested with restriction endonuclease at 37°C for 2-3 hours. Then, digested plasmids were analyzed by Agarose Gel Electrophoresis to observe the size of plasmid and insert. To verify the nucleotide sequence of insertion, samples were sent to the DNA sequencing.

SDS- Polyacrylamide Gel Electrophoresis and Silver Staining

SDS-PAGE was performed using the Mini-protein II Dual slab cell unit according to manufacturer's instruction. Discontinuous polyacrylamide gels (12% w/v separating and 4 % w/v stacking gels) were prepared. Following electrophoresis, gels can be silver stained to visualize bands. Gels were first fixed with fixative solution I containing 40 % (v/v) methanol and 10% (v/v) acetic acid for 30 min at room temperature followed by two 15 min incubations with fixative solution II which consists of 10% (v/v) ethanol and 5% (v/v) acetic acid. Gels then were incubation with 50 ml of 10 % (v/v) potassium dichromate and nitric acid containing oxidizer (BioRad) for 2 min and rinsed with Millipore water until cleared. After incubated with 50 ml of 10% (v/v) silver nitrate containing silver reagent for at least 30 min and rinsed twice with Millipore water, gels were developed with 100ml of 3.2% (w/v) silver stain developer including carbonate and paraformaldehyde. When the bands reached the desired density in relation to background, the reaction was stopped by 5% acetic acid.

Immunoblotting

The protein extracts of *M. paratuberculosis*, *M. smegmatis* and the kanamycin resistant recombinant clones were prepared by sonication and estimated following standard procedures (14, 15). The protein content of *E. coli* and the sonicate of mycobacterial cells were solubilized by boiling for 15 min in an equal volume of SDS-gel sample buffer

(Tris.Cl, pH 6.8, 2.5% SDS, 100 mM DTT, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue). The extracts were centrifuged for 15 min and the supernates were fractionated on 12% SDS-PAGE. A prestained protein molecular weight standard (Bio-Rad) was included in each SDS-PAGE gel, which were followed by transferred onto nitrocellulose membrane (Bio-Rad transblot medium) using a Bio-Rad Trans blot mini electrophoretic chamber (120 V, 80 A for 1 hour). Immunoblot filters containing fractionated proteins were blocked with 10% non-fat milk in tris-phosphate buffered saline (PBS) buffer at either 4°C overnight or room temperature for at least 3 hours. The membrane was washed with PBST twice for 5 minutes each and then incubated with rabbit hyperimmune anti-*M. paratuberculosis* serum (1:10,000) at 4°C overnight with gentle agitation. The membrane was again washed twice with PBST and incubated with 1:10,000 diluted Goat anti-rabbit IgG-peroxidase conjugates. After similar washes signal on the membrane can be visualized by adding opti-4CN diluent solution substrate (Bio-Rad). Following color development, filters were washed in distilled water and air-dried.

Unidirectional DNA deletion Exonuclease III

Purified pMptb#28 was digested with *XbaI* (Promega, Madison, WI) as described by the manufacturer. The recessed ends were filled in with α -phosphothiorate nt (Promega) and DNA polymerase as described by Sambrook et al. The sample was then digested with *SpeI* (Promega) to generate a 5' overhang closest to the insert, followed by phenol chloroform extraction and sodium acetate precipitation (51). The sample was added to an exonuclease buffer (0.66 mM Tris/Cl(PH 8.0), 66mM MgCl₂) and incubated at 37°C for 5min. ExonucleaseIII(2.5U) was added and 2.5ul aliquot were removed every 30 sec, mixed with 75ul of ice cold S1 reaction mixture (3mM potassium acetate, PH 4.6, 2.5mM

NaCl, 0.1 M EDTA and incubated at 50°C for 10min. DNA deletion was estimated by agarose gel electrophoresis (51). Re-circularization of new plasmids was done by T4 DNA Ligase (Promega) and transformation into *E. coli* TOP10 competent cells was performed by electroporation. Colonies were screened for smaller plasmids and subclones of pMptb#28 were isolated. For sequencing, each transformed colony was grown and plasmid DNA was isolated by use of Qiagen Midi Kit (QIAGEN). The T7 universal primer or Sp6 primer was used to sequence the inserts of the subclones. Immunoblotting was followed to check for producing the protein.

Construction of *E. coli* Subclone (EB486)

The sequence of 486 bp Opening Reading Frame producing 19kDa hypothetical protein in *Mycobacterium paratuberculosis* according to data base, was blasted to the sequence of 4.8kb insertion. After finding the area, the sequence of 486 bp was amplified and cloned to pBAD system as will describe in detail.

The Cloning System

The pBAD/His is a pUC-derived expression vector purchased from Invitrogen. It is designed for regulated, dose-dependent recombinant protein expression and purification in *E. coli*. The regulatory protein, AraC, was provided on the pBAD/His vector allowing regulation of pBAD. In the presence of arabinose, expression from pBAD was turned on while the absence of arabinose produces very low levels of transcription from pBAD. By varying the concentration of arabinose, protein expression levels could be optimized to ensure maximum expression of soluble protein. The plasmid pBAD/His is a 4.1 Kb prokaryotic expression vector. It has the following features: the pBAD promoter and the *araC* gene product for regulated expression of the gene of interest, N-terminal

polyhistidine tag for rapid purification of fusion proteins using ProBond™ resin, anti-Xpress™ epitope for detection of fusion proteins with the Anti-Xpress™ Antibody, enterokinase cleavage site to facilitate removal of the fusion protein, multiple cloning site in three reading frames to simplify subcloning in frame with the N-terminal polyhistidine tag, and ampicillin resistance gene and ColE1 origin for selection and maintenance in *E. coli*.

Oligonucleotide Primers design

According to the nucleotide sequence of on data base, a pair of oligonucleotide primers Forward and Reverse, including restriction recognition sites for *Bgl* II and *Eco*R I were designed to amplify exactly 486bp sequence.

Table 1: The Sequence of Primers Used in Construction of EB486 Subclone

Primers sequence	Target	Tm(°C)	Product
F 5'-CACCAG ATCTTCAGCTGT TGCAGGT -3'	Clone28	59	506bp
R 5'-CCACGA AATCGTGAAGCGTCAACTG-3'			

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 50 ul containing PCR reaction buffer, 3M Betaine , MgSO4 solution at a final concentration of 50mM, 10 mM of deoxynucleotides (dATP, dCTP, dGTP, dTTP); 3ul of each set of the primers; 1 unit of High Fidelity Taq DNA polymerase; and 1 ul volume DNA template of pMptb#28 plasmid carrying 4.8kb fragment. The reaction mixture was subjected to 30 cycles of

amplifications performed in a programmable thermal controller, as follows: 5 minute denaturation at 95°C, denaturation at 94°C for 45 seconds, 30 second annealing at 56°C; and 1 min primer extension at 72°C. After the 30 cycles, reactions were extended for 10 min at 72°C.

Purification of Amplified PCR DNA Product

In order to clean up the impurities, including primer dimmers, non-specific amplification products and templates, the PCR amplified DNA was purified by Wizard PCR Preps DNA Purification system (Promega). The PCR products were first separated by electrophoresis on 1% (w/v) low melting agarose gel (Fisher Scientific). The excised gel slice (around 300mg) was transferred to 1.5 ml microcentrifuge tube and heated at 70 °C until the agarose was completely melted. 1 ml resin was then mixed with melted agarose thoroughly and slowly passed through the minicolumn by 3-ml syringe. After washed with 2ml 80% isopropanol, DNA was eluted out with 50 ul sterile water at 9,600 rpm for 1min. Cleaned PCR product was further confirmed by sequencing.

Restriction Enzyme Digestion

Both pBAD/His plasmid and amplified DNA were digested by restriction endonucleases enzymes of *Bgl* II and *Eco*R I. Then, the digested plasmid pBAD/His was dephosphorylated by Shrimp alkaline phosphatase (SAP, Promega) for 1 h at 37°C to prevent self ligation. The shrimp alkaline phosphatase was inactivated by heating for 10 min at 70°C.

Ligation

The dephosphorylated pBAD/His vector (50 ng) was then ligated to an *Eco*RI and *Bgl* II PCR digest using T4 DNA ligase (Promega) following standard procedure. Using a ratio of 1:3 for the vector to genomic DNA digest, the ligation mixture was incubated at 16 °C overnight. As a control, a tube containing dephosphorylated pBAD/His vector only was treated under the same conditions.

Electroporation in *E. coli*

The mixture of ligated pBAD/His and digested amplified product was transformed into *E. coli* TOP10 competent cells by electroporation as described by Sambrook et al.

Electroporation was performed using the Gene Pulser and Pulse Controller instruments from Bio-Rad (Bio-Rad Laboratories, Richmond, CA). A volume of 2 ul of the ligated DNA was mixed with 50 ul of electrocompetent *E. coli* TOP10 cells. It was placed on ice for few minutes and then the cells were transferred into an ice cold 0.2 cm electrode chamber (electroporation cuvette). Electroporation was performed at 1.75 KV, 200 (ohms), and 2.5uF using the standard setting. The electroporated cells were suspended in 1.0 ml of SOC medium, incubated for 1 hr at 37 °C, and then spread onto LB agar plates containing ampicillin (50 ug/ml). The plates were incubated at 37 °C for overnight. pBAD/His vector was transformed under the same conditions as a positive control. The colonies were further screened and verified by Cell Pop PCR described later.

Verification of EB486 Cloning using Cell Pop PCR Method

The PCR reaction was performed in a total volume of 25 ul containing PCR reaction buffer, 3M Betaine , MgSO₄ solution at a final concentration of 50mM, 10 mM of

deoxynucleotides (dATP, dCTP, dGTP, dTTP); 1.5ul of each set of the primers; 0.5 unit of High Fidelity Taq DNA polymerase; the colonies were randomly picked from the agar plate by sterile toothpick and directly served as DNA template without DNA extraction. The reaction mixture was subjected to 30 cycles of amplifications performed in a programmable thermal controller, as follows: 15 minute denaturation at 95 °C, denaturation at 94°C for 45 seconds, 30 second annealing at 56 °C; and 1 min primer extension at 72 °C. After the 30 cycles, reactions were extended for 10 min at 72 °C. The amplified products were visualized by agarose gel electrophoresis.

Screening of EB486 for protein expression

The PCR positive subclones were grown, induced with L- Arabinose and harvested as described before. Each cell pellet resuspended in 25 ul of sterile deionized water and 75 ul sample buffer were solubilized at 95°C for 15 minutes and fractionated on 12% SDS-PAGE gel followed by transferred onto nitrocellulose membrane (Bio-Rad transblot medium) at 4°C for 12-16, voltage of 40V using a Bio-Rad lectrophoretic chamber. A prestained protein molecular weight standard (Bio-Rad) was included in each SDS-PAGE. Immunoblot filters containing fractionated proteins from recombinant *E. coli* were blocked with 1% BSA in PBST buffer at either 4 °C overnight or room temperature for 3 hours. Then the membrane was incubated with anti-His or anti-Xpress™ monoclonal antibody (Invitrogen) (1:4000) at 4 °C overnight with gentle agitation. The membrane was followed by incubating with 1:4000 diluted Goat anti-mouse IgG-peroxidase conjugates. The signals on the membrane can be visualized by adding opti-4CN diluent solution substrate (Bio-Rad)

To optimize overexpression of recombinant protein, the recombinant colony was inoculated and grown in Laura Britani (LB) broth media containing 50 ug/ml ampicillin at 37 °C with agitation at 175 rpm until OD₆₀₀ equals 1.5. The 100ul overnight culture were then transferred to each 10ml LB broth and grown in the same condition until OD₆₀₀ was 0.5. A serial dilution of L-arabinose (Becton Dickinson, Sparks, MD) at a final concentration from 0.0002% to 2% was added into each 10 ml culture and 1ml sample was taken every two hours from each tube. The aliquot of cells from each tube was centrifuged at 12,000rpm for 2 minutes and kept frozen at -20 °C for further analysis by immunoblotting as describe earlier.

Purification of Recombinant Protein

6x His-tagged fusion proteins were purified by using B-PER 6xHis Spin Purification Kit (PIERCE Biotechnology) with some modification as manufacturer described. The bacterial cell pellet from a 250ml culture (OD₆₀₀ = 1.5.-3.0) was re-suspended in 10ml B-PER reagent by vortexing until the cell suspension was homogeneous. The homogenous mixture was gently shaken at room temperature for 10min. The separation of soluble from insoluble proteins was followed by centrifugation (14000 rpm, 15min) at 4°C in autoclaved, high-speed centrifuge tubes. The supernatant (soluble fraction) was transferred to a 15 mL capped conical centrifuge tube; a small sample was reserved for immunoblot analysis. The pellet was also stored as the insoluble fraction. The Nickel-Chelated Agarose was equilibrated at room temperature and 1 mL of the 50% gel slurry was added to the soluble fraction, and was incubated for 10 minutes at room temperature, then centrifuged at 6,000 rpm for 10 minutes at 4°C. The supernatant was removed and

stored at -80°C as Flow Through and the resin was re-suspended with 0.25 mL of Wash Buffer, also equilibrated at room temperature. The gel-bound, 6x-His fusion protein was transferred to the Microfilter Spin column and centrifuged for 3 minutes at 10,000 rpm at 4°C. The Microfilter spin column was transferred to a new autoclaved collection tube after each centrifugation. The agarose slurry was re-suspending in 0.5 mL of wash buffer and centrifuged at 10,000 rpm for 3 minutes following an incubation of 5 minutes. During the incubation, the collection tube gently tapped to re-suspend the slurry, and column was washed 5 more times. All the washes were collected in separate collection tubes: Wash1-Wash6. The resin was transferred to a new Microfilter Spin column and was incubated in 0.5 mL of Elution Buffer for 5 minutes, followed by centrifugation at 12,000 for 3 minutes. This step was repeated 4 more times and the samples were collected into separate collection tubes: Eluted1-Eluted5. All the fractions were stored at -80°C for further analysis.

Construction of *M. smegmatis* Subclone (SM486)

In order to clone the sequence of 486 bp Opening Reading Frame producing 19kDa protein to *Mycobacterium smegmatis*, an *E. coli* mycobacterium shuttle vector, Pmip12, was used in this experiment (36). The sequence 486 ORF was amplified and ligated to Pmip12 shuttle vector and transformed in *E. coli* top 10 competent cells and then transformed to *M. smegmatis* mc2155 competent cells as will describe in detail. According to the shuttle vector map, two different restriction enzymes, *BamH I* and *Kpn I* were chosen to use for directional ligation. Therefore, a set of two primers were designed to amplify the 486 bp of ORF sequence.

Table 2: Sequence of Primers Used in Construction of SM486 Subclone

Primers sequence	Target	Tm (°C)	Product
F-5'ATCGGGATCCGTGAAGCG TCA ACTGACGATC3' R-5'ATCGGG TACCTCAGCTGTTGCAGGTCACATC3'	Clone28	64	506bp

Cloning the Plasmid Construct into *E. coli* (The Transition Step)

PCR was performed by using the oligos as mentioned before. Then PCR product and shuttle vector were digested with *BamH I* and *Kpn I* restriction enzyme and ligation procedure was performed as described earlier. Electroporation to *E. coli* TOP 10 competent cells, screening the subclone by Cell Pop PCR, and confirming the right Subclone by plasmid extraction was achieved as previously stated.

Preparation of *M. smegmatis* mc2155 Competent Cells

One single colony of *M. smegmatis* mc2155 was inoculated in 2ml Middlebrook 7H9 broth supplemented with OADC enrichment (Difco Laboratories, Detroit, MI), 0.5% glycerol and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo) at 37° C with constant shaking. After 2 days, 2 ml were transferred to 20 ml of the same media, and again after 2 days 20 ml were transferred to 1 liter of the media and incubate until OD₆₀₀ achieves 0.2 to 1 in 2 days. Then it was incubated on ice for 1.5 hours before harvesting. The cells were harvested in 250ml centrifuge bottles by spinning for 10min at 5,000 x g at 4° C and the supernatant was discarded. The pellet was resuspended in an equal volume (250ml) of 10%glycerol, and centrifuge again. The next was resuspending each

pellet in 10ml ice-cold 10%glycerol, combining cells and transferring to two 50ml tubes. The volume of each tube was brought to 50ml with ice-cold 10%glycerol and centrifuged for 10min at 2,000 x g at 4° C. The pellet was re-suspended in 50ml ice-cold 10%glycerol and then centrifuged as above for 15min. Each of the two pellets was re-suspended in 10%glycerol for a final volume of 1ml. At this time, cells were aliquot about 100ul into 1.5ml sterile tube and stored at -80°C to use later

Electroporation of *M. smegmatis* Competent Cells

M. smegmatis mc2155 cells were prepared for electroporation following the procedure as described earlier by Jacobs *et al.*(1991). Electroporation was performed using the Gene Pulser and Pulse Controller instruments from Bio-Rad (Bio-Rad Laboratories, Richmond, CA). The electroporated cells were suspended in 1.0 ml of Middlebrook 7H9 broth supplemented with OADC enrichment, 0.5% glycerol and 0.05% Tween 80, incubated at 37 C with agitation (150 rpm) for 2 h, and then spread onto 7H10 agar with 50 ug/ml kanamycin. The plates were incubated at 37 C and colonies were counted after 72 h.

Bioinformatics Analysis

In this study some bioinformatics software used are including: Blast analyses, ORF finder, Oligonucleotide Properties Calculator, Primers design, and Expasy Proteomics tools for protein identification and characterization.

<http://www.ncbi.nlm.nih.gov/BLAST/>

http://bioinformatics.org/sms/orf_find.html

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

<http://www.bioinfo.rpi.edu/~zukerm/>

<http://www.basic.northwestern.edu/biotools/oligocalc.html>

<http://www.expasy.org/tools/>

Purification of Recombinant Proteins from Polyacrylamide Gels

Purification of expressed proteins from *E. coli* and *M. smegmatis* hosts were performed following standard procedures (51, 15). Initially two SDS gels loaded identically with samples from marker and bacterial cells were prepared. One gel was stained with silver stain following standard procedure as described earlier. Protein bands with size of interest were identified and then lined up with the second unstained gel. Portion of the gels with bands of interest were cut, transferred to sterile tubes and then incubated with elution buffer. The remained unstained gel was then stained with silver stain in order to confirm the excision of the correct part of the gel. The excised gel pieces placed in sterile microcentrifuge tube was covered with elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) and incubated in a rotary shaker at 30 °C for overnight. The supernatant was transfers to new tube following centrifugation $10,000 \times g$ for 10min. Aliquots of eluted protein were then confirmed by sliver stain and with rabbit anti-MAP IgG antibodies.

Carbohydrate analysis:

Due to the variation in the size of the expressed protein in lystaes from *E. coli* and *M. smegmatis* recombinant cells which may be due to posttranslational modification, we sought to investigate whether the modification of the protein is due to glycosylation or something lese. Evaluation of the protein for glycosylation was performed by ConA staining and by the use of deglycosylation enzymatic assay.

A: ConA Staining

The procedures for staining of nitrocellulose blots with peroxidase-conjugated concanavalin A (ConA) were basically identical to those used for immunoblotting as described earlier. Nonspecific binding was blocked by incubating blots at either 4 °C for overnight or at room temperature for at least 3 hours with 4% (wt/vol) BSA in phosphate-buffered saline (PBS) with Triton X-100 (0.2%, vol/vol). Following several washes with PBS and PBS-Triton X-100, the blots were incubated with 20 ml of ConA-peroxidase conjugate (0.2 purpurogallin units per ml;Sigma) in 2% (wt/vol) BSA in PBS-Triton X-100 for 4 °C for overnight or at room temperature for 5-7 hours as described by the manufacturer. Following several washes, the membrane was developed and evaluated for glycosylation using opti-4CN diluent solution substrate (Bio- Rad).

B: Enzymatic Protein Deglycosylation assay

Enzymatic analysis for the presence of glycoprotein in the samples was performed using Deglycosylation kit purchased and following instructions provided by the manufacturer (EDEGLY Sigam-Aldrich). Briefly 10ul of 5x Reaction Buffer and 2.5ul of Denatuation Buffer were added to 35 ul of bacterial cell extracts including 100mg or less of protein and then heated at 100°C for 5min. After cooling at room temperature, 2.5ul of TRITON X-100 solution was added to each sample, and followed by adding 1 µl each of the PNGase F, O-Glycosidase, α -2(3, 6, 8, 9) Neuraminidase solutions, β (1-4)Galactosidase, and β -N-Acetylglucosaminidase. The mixtures were incubated for 3 hours at 37 °C, and then analyzed by similar Staining or Immunoblot. Bovine fetuin was supplied as a control glycoprotein since it contains sialylated N-linked and O-linked oligosaccharides

Evaluation of Expressed protein for Antigenicity

The variation in size for the expressed protein originated from *E. coli* and *M. smegmatis* recombinant clones hinted to us to investigate for the presence of any immunomodulatory interference. Initially, the same protein from the two different hosts was evaluated against rabbit anti-MAP IgG antibodies. Then the proteins were also evaluated against T- cells from two healthy individuals and two patients with Crohn's disease.

Source of Lymphocytes

In this study, mononuclear cells (mostly T lymphocytes) originated from two individuals that were considered healthy controls and two patients with confirmed Crohn's disease diagnosis. Blood cells were collected in EDTA-tubes and transported to the lab within 24 hours. Immediately, the blood samples were processed for isolation of peripheral blood mononuclear cells (PBMNC's) and then used in T-cell proliferations assays.

Isolation and Quantitation of Human PBMCs

PBMC were isolated using lymphoprep density gradient separation medium following the manufacturer's procedure (Greiner Bio One, Inc). Fresh 5ml blood sample were immediately processed in a Biosafety Cabinet Class II as follows: First, blood samples were diluted 1:1 (v/v) by RPMI culture media (Invitrogen, CA) at room temperature. And then, a volume of 4mL of diluted blood was layered on 4mL Lymphoprep solution in 15mL polypropylene tubes. Tubes were centrifuged at 1,550 rpm in room temperature for 30 min. As a result of centrifuge, the density gradient interface forms, this was followed by transferring the white band containing PBMCs to a sterile tube. Hemocytometer counting was used to determine the PBMCs concentration.

T-Cell Proliferation Assay

Isolated PBMC adjusted at final concentration of 1×10^5 cells/ well in RPMI 1640, and were mixed with 10% plasma. T- cells were then evaluated against mitogen controls including PHA (phytohemmatoagglutinin) and PWM (Polkweed mitogen). PPD from MAP was prepared in our lab and was also used. Additionally protein lysates from recombinant *E. coli* and *M. smegantis* were used in this assay. The cell extracts of *E. coli* and *M. smegmatis* and MAP PPD were prepared by sonication of cell pellets from cultures grown (approximately six weeks for MAP, 2-3 days for *M. smegmatis* and 1 day for *E. coli*) followed by centrifugation at 16,000 rpm for 30 m, Cell debris were discarded and supernatants were transferred to sterile tube and stored at -80°C until use. Protein content was measured in each sample following Bradford method. (6)

Initially, in order to determine the optimal concentration, bacterial extracts (*E. coli* and *M. smegmatis*) and MAP PPD were added to triplicate wells at different concentration of 2, 20, 40, 80 ug/ml. The plates were then incubated at 37°C in a humidified air with 5% CO_2 incubator during 3 days. The proliferation assay was followed by labeling the PBMC with bromodeoxyuridine (BrdU, Roche Molecular Biochemicals, IN) and reincubated for additional 24 h, and cell transformation was then measured using cell proliferation ELISA BrdU commercially available kit from Roche ((Indianapolis, IN). Briefly, BrdU was taken up by DNA of the cells while they were dividing and then was measured by using a monoclonal antibody, anti-BrdU-POD, conjugated with peroxidase. The immune complexes were detected by a substrate reaction and the reaction product was quantified by measuring the absorbance at 492nm in an ELISA Auto Reader II (Ortho Diagnostic Systems, Inc). The positive controls with the optimal concentration

were including: PHA (20ug/mL, Sigma, MO), PWM, pokeweed mitogen, (10ug/ml Sigma, MO), and MAP purified protein derivative (PPD; 5ug/mL). The cells only were used as negative control.

CHAPTER THREE

RESULTS

Preliminary data for Clones pMptb#28 and Smeg19k

Previously in our lab, a genomic library of *Mycobacterium avium paratuberculosis* was constructed in *E. coli*. Colonies were screened for gene expression using immunoblot and rabbit anti-*M. paratuberculosis* antibody. Clones specific to *M. paratuberculosis* were identified when they reacted negatively with rabbit anti-*M. tuberculosis*, rabbit anti-*M. leprae* and rabbit anti-*M. bovis* and rabbit anti-*M. avium* subspecies *avium* antibodies. pMptb#28 recombinant clone contained the insert of 4.8 kb and encoded for a single protein of 16kDa. Surprisingly, *M. paratuberculosis* lacked a 16 kDa protein band when screen with rabbit anti-*M. paratuberculosis* antibodies. When the 4.8 kb *BamH I* fragment was cloned in *M. smegmatis* using a shuttle plasmid, it produced a 19 kDa protein (Smeg19k) similar to the size observed in extracts from *M. paratuberculosis*. These preliminary data were collected earlier in our lab.

Confirmation of the Identity of Clones pMptb#28 and Smeg19k Plasmids

In order to confirm the identity of the clones pMptb#28 and Smeg19k, both clones were inoculated from stock culture and then cultured in LB with ampicillin and 7H9 with kanamycin, respectively. Cell pellets were analyzed at both the DNA and protein levels.

DNA Level:

DNA extracted clones pMptb#28 and Smeg19k were digested with *Bam*HI restriction endonuclease enzyme. The presence of 4.8 kb insert on 1% agarose gel is shown in Figures 1 and 2.

Protein Level:

Protein extracts from pMptb#28 and Smeg19k clones and from *M. paratuberculosis*, *M. smegmatis* transformed with pNEZ6.3 shuttle plasmid and *E. coli* with pcDNAII plasmid were screened by immunoblot using *E. coli* adsorbed rabbit anti-*M. paratuberculosis* antibodies. As shown in Figure (3), a 16kDa protein band was detected in the pMptb#28 (lane 5) compared to a 19kDa protein band detected in the protein extracts from Smeg19k clone (lane 3) similar to the native size detected in the *M. paratuberculosis* protein extract (lane 2). There was no reactivity in extracts from the hosts, *E. coli* (lane 6) or *M. smegmatis* (lane 4) without the insert.

Identification of 16/19 kDa Encoding Sequence (ORF):

By DNA Nested Deletion:

The 4.8 kb insert was evaluated and analyzed for the presence of the possible ORF encoding the 16/19 kDa expressed protein. Initially the 4.8 kb insert was sequenced from both ends (the 5' and 3') but since the MAP genome sequence was not published yet, alternative methodology were used in attempt to identify the ORF. Consequently, DNA nested deletion (Gene Walking) was employed. Following unidirectional deletion of linearized pMptb#28 by *ExonucleaseIII*, minilibrary of subclones were screened with rabbit anti-*M. paratuberculosis* antibody. The goal was to find a subclone with the

smallest plasmid that still able to produce the 16kDa protein in *E. coli*. As shown in Figure (4), the size of the plasmid is getting smaller with increased exposure to Exonuclease. Some of these plasmids were selected and relegated then transformed into *E. coli* TOP10 competent cells. Subclones were examined for expression of 16 kDa protein. As shown in Figure 5, some subclones expressed the expected 16 kDa protein and others did not. The subclones were analyzed for determination of plasmid size. Figure 6 illustrates that subclone #4 contained the smallest insert (2.5 kb) which continued to the 16kDa protein.

By Nucleotide Sequencing:

The extracted plasmid from subclones #4 and #5 were analyzed by sequencing. Nucleotide sequences from the 5' and 3' ends of the two subclones and the release of the *M. paratuberculosis* genome sequence at this time have enabled us to identify the region on the 4.8 kb insert which may encode for the expression of the 16/19 kDa protein. Blast analysis of the identified sequence region with the data base has produced identity with a 19 kDa protein from *M. bovis* and *M. leprae*. Accordingly, we were able to identify the start codon and the stop codone of the possible ORF consisting of 486 bp.

Construction of Subclone EB486 (*E. coli* Subclone)

Given that the unidirectional exonuclease technique was capable of eliminating some additional sequences that are unrelated for the expression of 16/19 kDa protein, the technique was insufficient for identifying the exact open reading frame that codes for the protein. Therefore it was required to find the exact ORF of the protein, and then to expresses in *E. coli* and *M. smegmatis* to able to do further characterization. The 486bp sequence of ORF was identified using bioinformatics software. Oligonucleotide primers

with cloning enzyme sequence for *Bgl* II and *Eco*R I were designed and the possible ORF was amplified using PCR technology from pMptb#28 DNA template. The PCR product was purified, ligated to pBAD/His expression plasmid vector and then transformed into *E. coli* competent cells. As shown in figure (7), the expected size of 506 bp PCR product was detected on 2% agarose gel. Controls with templates from pcDNAII were negative. The amplified ORF was further confirmed through nucleotide sequence analysis. The amplified ORF sequenced was purified and then ligated into dephosphorylated pBAD/His plasmid and then electroporated into TOP 10 *E. coli* competent cells. Ampicillin resistant colonies were analyzed for the presence of the insert and the expression of encoding product.

Identification of Recombinant Subclone EB486

Three ampicillin resistant colonies were picked, subcultured and then analyzed for the presence of the 486 bp ORF sequence and the expression of 16/19 kDa protein. As shown Figure (8), all three colonies were positive by Cell Pop PCR, PCR product was purified and sequenced and was confirmed to contain the expected 486 bp sequence. Extracted plasmid from positive colonies were further digested with both enzymes, *Bgl* II and *Eco*R I, and then analyzed on agarose gel. As shown in Figure (9), the insert with an estimated 486bp was observed. The three colonies were inoculated in LB broth and induced by L-arabinose to express the fusion protein, which were further examined by immunoblot using anti-His antibody, anti-Xpress antibody and *E. coli* adsorbed rabbit anti-*M. paratuberculosis* antibody. As shown in Figures 10, 11 and 12, all colonies expressed 22 kDa recombinant proteins including the additional 6kDa His-tag/Xpress tag

as described by the manufacture. One of the colonies labeled EB486 was subcultured and stored.

Over expression of Recombinant Protein EB486

In order to get optimal expression of protein EB486, three conditions were considered including culture temperature, incubation time, and variable concentrations of L-arabinose. L-arabinose was used as the inducer for EB486 in the pBAD system. The different culture temperatures included 25°C and 37°C didn't show any differences in protein expression. Hence, optimal growth was achieved with either temperature. The pilot study was performed by growing the EB486 single colony in LB broth until an optical density (O.D.600) of 0.5 and then different concentration of L-arabinose was induced in different time of incubation of 0hr, 2hr, 4hr, 6hr, 8hr, and overnight. This study, incubation time was found to be directly proportional to protein expression. However, SDS gel analysis of EB486 expression of 8 hours and overnight appeared to show more background and the presence of none specific bands. Thus, 4 to 6 hours incubation time after induction was preferred for optimal expression of the protein. In the case of induction by L-arabinose, varying concentrations at .0002%, 0.02%, 0.2%, and 2.%, were used to determine optimal expression. The concentration of 0.02% was selected based on the quantity and quality of expression as shown in figure. Figure 13 illustrates the optimal expression of the 16 kDa protein from subclone EP486.

Purification of Recombinant Protein EB486

The recombinant protein EB486 includes N-terminal polyhistidine tag and an additional encoding sequence named Xpress as described by the manufacturer. The estimated product of the pBAD protein product is 6 kDa. The presence of anti-His and anti-Xpress monoclonal antibodies have facilitated in the identification and purification processes. As shown in Figure (14), the 22 kDa (including 6 kDa from the vector) was purified which then used in further experiments.

Construction of Subclone MS486 (*M. smegmatis* Subclone)

According to the multiple cloning sites (MCS) of the shuttle vector, two different restriction enzymes, *Bam*H I and *kpn* I were selected. Consequently, a new set of oligonucleotide primers were designed and added to the 486 ORF sequence in order to be ligated the shuttle plasmid and then ultimately transformed into *M. smegmatis* competent cells. Using pMptb#28 DNA template, the 506 bp (486 ORF sequence plus cloning enzyme sequence) was amplified by PCR, purified and then ligated into linearized shuttle plasmid. To facilitate cloning into *M. smegmatis* competent cells, a transitions step was required. Therefore, Ligation mixture first was transformed into *E. coli* competent cells. Cloned plasmid was verified and then electoporeted into *M. smegmatis* competent cells. As shown in Figure (16), digested plasmid from the recombinant colony#5 revealed the presence of an estimated 506 bp insert. DNA extracted from colony #5 was further confirmed by sequencing. After confirmation, purified extracted DNA from *E. coli* colony#5 was electroporated to *M. smegmatis* competent cells. After 3 days many colonies were observed. Figure (17) shows recombinant *M. smegmatis* colonies after 6

days. Four of those colonies were grown in 2ml 7H9 broth in 2 days and then transformed to 20 ml culture, then was sonicated to obtain protein extracts as described earlier. At the protein level, protein extracts from *M. smegmatis* transformed with pNEZ6.3 (as negative control), *M. smegmatis* transformed with pShuttle19K and four new *M. smegmatis* transformed were screened against *M. smegmatis* adsorbed rabbit anti-*M. paratuberculosis* antibody. In Lane 1, a 19kDa antigen band was detected in the protein extract of *M. smegmatis* recombinant harboring pShuttle19kDa similar to the native size detected in the *M. paratuberculosis* protein extract as discussed earlier. Lanes 3,4,5 and 6 represent those new *M. smegmatis* recombinant colonies expressing 19kDa protein. There was no reactivity in extracts from the host *M. smegmatis* (lane 2). The expressed protein from the 486 ORF is 19 kDa compared to 16 kDa in *E. coli* host.

Estimation of the 486 bp ORF Encoding Product

The 486 bp ORF was translated and each amino acid molecular weight was determined. Overall the actual calculated size of the peptide sequence encoded on the 486 bp is 15.6 kDa. This protein size is similar to that expressed in EB486 (in *E. coli* host) and smaller than in *M. smegmatis* (SM486).

Analysis of SM486 for Posttranslational Modification

The difference in the expressed product of the 486 bp ORF in both *E. coli* and *M. smegmatis* (16 kDa versus 19 kDa) had suggested that *M. smegmatis* may support posttranslational modification. We attempted to analyze the SM486 protein for posttranslational Glycosylation based on some homologous identity with 19 kDa from *M. tuberculosis* in the data base and the fact that *E. coli* does not support posttranslational glycosylation.

Evaluation of SM486 protein for Carbohydrate Content:

By Enzymatic Deglycosylation

The simplest way to show the presence of carbohydrate on glycoproteins is by removing the carbohydrate and then recognizing the mobility shift on SDS-PAGE gels and this was done by using an enzymatic deglycosylation kit (E-DEGLY kit from Sigma-Aldrich).

The enzymatic method can remove the carbohydrates without any protein degradation and this is an advantage over the chemically removing the carbohydrates from glycoprotein. The E-DEGLY kit contains all enzymes (PNGase F, Neuraminidase, O-Glycosidase, Glycosidase, N-Acetylglucosaminidase) require to completely remove all N-linked and O-linked carbohydrates from glycoprotein. In this experiment, Bovine fetuin was used as positive control, which contains N-linked and O-linked oligosaccharides.

As shown in figure (20) lanes 1 and 2 contain Bovine fetuin glycoprotein before and after deglycosylation, respectively. It was clear that there was a shift in the size of the protein before and following the deglycosylation treatment. The observation confirms the validity of the purchased kit to be used for evaluation of glycoproteins. When the same

experiment was applied on protein extracts from the recombinant *M. smegmatis* expressing the 19 kDa protein, there was no shift in protein size (Figure 20, lanes 3 and 4). Protein extract from the recombinant *M. smegmatis* was evaluated for possible inhibitors. In this experiment, protein extract from the recombinant *M. smegmatis* was spiked with Bovine fetuin glycoprotein control and then subject to enzymatic deglycosylation. As shown in Figure 20. The Fetuin glycoprotein has shifted in size as expected following the treatment illustrating absence of any inhibitors. The 19kDa protein was not affected by the deglycosylation treatment (Figure 20, lane 5)

By ConA Staining:

The possible lack of glycosylation in the 19kDa protein was further evaluated by ConA staining. ConA is a lectin that specifically binds to alpha-D-glucose and alpha-D-mannose. The protein samples analyzed earlier in Figure 20 were also evaluated following staining an immunoblot with peroxidase-conjugate ConA. As shown in Figure 21, the Fetuin glycoprotein (positive control) stained positively with ConA confirming the presence of ConA even following the deglycosylation treatment due to presence of remnant carbohydrates on this protein. However and most importantly, the 19kDa protein was absent in the immunoblot stained with ConA. The observation was true for the 19 kDa before and after the deglycosylation treatment and for eluted recombinant 19kDa protein from SDS PAGE.

Evaluation of EP486 and SM486 proteins for T-cell Proliferation Response:

In this experiment two mitogens, PHA and PWM were supplied as positive controls. PHA is a plant mitogen and measures the overall function of lymphocyte responsiveness. In fact, PHA stimulates directly proliferation of peripheral blood mononuclear cells by

mechanisms different than those antigen processing and presentation to lymphocytes. In this study, PHA was used at the final concentration of 20ug/ml, which has been identified in our lab to present optimum lymphocyte proliferation. Another positive control, PWM, (Pokeweed Mitogen) was used to evaluate the proliferation response of T-lymphocytes independent to other cells such as B-lymphocytes and other antigen presenting cells. PWM was used at final concentration of 10ug/ml.

Earlier experiments illustrated that the 486 bp encoding sequence of the 19 kDa protein had expressed a 16 kDa in *E. coli* and as 19 kDa in *M. smegmatis*. The protein expressed in both bacterial hosts regardless in the difference in molecular weight reacted similarly to rabbit-anti-MAP IgG antibodies. In these experiments, the protein from the two bacterial hosts was evaluated against T lymphocytes from healthy individuals and patients with Crohn's disease. Protein extracts from *E. coli* without the gene and *M. smegmatis* without the gene were also included. Additionally, purified protein from the two hosts was also included.

The proliferative response of PBMC samples isolated from Crohn's patient and healthy individuals were quantitatively measured and compared. Preliminary data indicate that there is no significant difference in the proliferative ability of this protein expressed in *E. coli* compared to *M. smegmatis* (Table 4). T-cells from patients with Crohn's disease proliferative response to PHA clearly indicate a decrease in response when compared to T- cells from normal controls (Table3, Figure 22).

CHAPTER FOUR

DISSCUSSION

Vaccines are the most cost-effective medical intervention known to prevent disease. The urgency for development of an appropriate multivalent vaccine vehicle against mycobacteria is mounting. This is due to the increasing incidence of multiple drug resistant (MDR)-*M. tuberculosis* strains (49, 18) and the ineffectiveness of current therapies for the treatment of disseminated disease in AIDS patients and other mycobacteriosis (48). Discovery of effective vaccines require identification of appropriate antigens and their ability to modulate the immune response causing acquired protective immunity. Proteins can be modified through glycosylation, phosphorylation, acylation or by other mechanisms which ultimately have an impact on their antigenicity. Cloning of some mycobacterial protein in *E. coli* may lead to expression of proteins with smaller molecular weight than expected due to that fact that *E. coli* supports limited posttranslational modification. Consequently, mycobacterial genes should be cloned in similar hosts that may aid in the expression of the expected native proteins. Shuttle plasmids are needed in order to accomplish such mission. Many efforts have been expended in the construction of an appropriate shuttle vector that may be used as a cloning vector to study mycobacterial genes or as a vehicle for the development of a suitable vaccine. The first attempt to form an *E. coli-Mycobacterium* shuttle plasmid vector was initiated by constructing a chimeric shuttle plasmid containing mycobacteriophage DNA inserted into an *E. coli* cosmid (29). This shuttle plasmid replicated in *E. coli* as a plasmid and in mycobacteria a phage. This was followed by the construction of pIJ666 by Snapper *et al.* (1988). The application of these shuttles in

mycobacterial genetics was limited to gene transfer with poor transfection/transformation efficiencies and no record of expression at the protein level. Nevertheless, this was an important step forward in gene analysis in mycobacteria. Radford *et al.* (1991) constructed the shuttle plasmid pEP3 with a size of 4.5 kb, but the transformation frequency was comparable to those obtained by Snapper. Lazraq *et al.* (1991) constructed the shuttle plasmid pDC100 with a molecular size of 10.4 kb. pDC100 appeared to lack unique cloning sites and it replicated in mycobacteria at low copy number that hampered its analysis on agarose gel. Hinshelwood *et al.* (1992) constructed pMSC1 which apparently was suitable only for construction of a genomic library or biosynthetic pathways analysis. pMSC1 is a 13.1 kb and contained unique sites for BamHI and HindIII only. In our laboratory, pNEZ6.3 was designed to overcome the limitations of other shuttle vectors. pNEZ6.3 vector is a phagemid that can be used either as a plasmid or a phage. In addition, the size can be reduced without loss of any functions. pNEZ6.3 contains two multiple cloning sites downstream of the T7 and the Sp6 promoters that enable further detailed analysis of multiple genes in one host. These promoters facilitate in vitro transcription using T7 or Sp6 RNA polymerase and the synthesis of an mRNA strand of the cloned gene that may be labeled and used as a riboprobe. They are also useful for sequence analysis using one of several primers such as T7, Universal, Sp6 or Reverse primer. The presence of F1 *ori* enables the rescue of ssDNA of the cloned gene after infection of the *E. coli* host with an appropriate phage such as M13. Since mycobacteria are susceptible to kanamycin, the drug resistant marker encoded on pNEZ6.3 shuttle vector facilitates screening of recombinants in mycobacteria . pNEZ6.3 expressed the kanamycin resistant gene in *M. smegmatis* whether kanamycin

was present or absent in the media. This is contrary to report by Lazraq *et al.* (1991) that pDC100 shuttle was lost when *M. smegmatis* was grown in media without kanamycin. Previously in our lab, genomic library of *Mycobacterium avium paratuberculosis* was constructed in *E. coli*. Colonies were screened for gene expression using immunoblot and rabbit anti-*M. paratuberculosis* antibody. pMptb#28 recombinant clone contained and insert of 4.8 kb and encoded for a single protein of 16kDa. Surprisingly, *M. paratuberculosis* lacked a 16 kDa protein band when screened with rabbit anti-*M. paratuberculosis* antibodies. When the 4.8 kb BamH I fragment was cloned in *M. smegmatis* using a pNEZ6.3 shuttle plasmid, it produced a 19 kDa protein (Smeg19k) similar to the size observed in extracts from *M. paratuberculosis* (Figure 3). The observation using the shuttle technology suggested a possible posttranslational modification in mycobacteria. In order to verify that the expression of 16/19 kDa protein in the two different hosts is not due to switching in the starting codon, we analyzed the 4.8 kb insert for the presence of the encoding sequence ORF. Earlier in the study, the *M. paratuberculosis* genome sequence was not published yet so sequencing the both ends of the 4.8 kb insert did not reveal any breakthrough. Performing Unidirectional Deletion using ExonucleaseIII allowed us to identify the region with possible ORF (Figure 4,5,6). At this phase of the study, the *M. paratuberculosis* genome sequence was revealed. The ends of the new subclones and using BLAST analysis with *M. tuberculosis* 19 kDa encoding gene resulted in the identification of 486 bp in the 4.8 kb insert. The sequence of 486 of ORF is including starting codon GTG and stopping codon TGA. . The 486 is coding 161 amino acids, which has molecular weight of 15.6kDa. When the 486 bp sequence of ORF cloned in *E. coli*, the expressed protein in *E. coli* without vector

encoded peptide was estimated at 16kDa. However, when the same 486bp of ORF cloned in *M. smegmatis*, the expressed protein was 19kDa. These results clearly showed that *M. smegmatis* is able to produce 3 kDa more than the calculated size of the protein according to the exact sequence of ORF. The difference in the sizes of the expressed protein may be related to posttranslational modification. The lack of possible phosphorylation, glycosylation or other posttranslational modification of the 19 kDa protein in the *E. coli* host can result in the formation of a protein with smaller mass. This observation was also reported by Garbe *et al.* (1993) while analyzing a 19 kDa antigen of *M. tuberculosis* in *M. smegmatis*. In their experiment, a DNA fragment encoding the *M. tuberculosis* 19 kDa protein was cloned into a shuttle vector and then transformed into *E. coli* and *M. smegmatis*. The expressed protein was 16K in *E. coli* and 19K in *M. smegmatis*. The difference the sizes was due to the lack of glycosylation in *E. coli*.

The lack of complete expression of both the 19 kDa protein of *M. paratuberculosis* and the 19 kDa protein of *M. tuberculosis* in the *E. coli* system is of concern to investigators and underscores the necessity to study mycobacterial virulence antigens in a homologous host. The role of posttranslational modification in expressed proteins especially in immunomodulation is yet to be elucidated.

Posttranslational modification has been investigated vigorously in recent years.

Consequently, many glycoproteins (N or O-linkage) have been reported in both archaeobacteria and eubacteria (3,7,47), which defeated the dogmatic belief that prokaryotes are unable to support posttranslational modifications such as glycosylation.

This includes identification of glycoproteins *Neisseria meningitidis* (57), *Flavobacterium meningosepticum* (45,50) *Streptococcus sanguis*(17), *Bacillus alvei* (39), *Clostridium* spp.

(22,38), *Bacteroides cellulosolvens* (22), *Thermoanaerobacter thermohydrosulfuricus* (5) and *Mycobacterium tuberculosis* (12,13,7). In this study, the data clearly illustrates that *M. paratuberculosis* supports posttranslational modification (Figure 3).

This study focused on glycosylation as a possible mechanism for posttranslational modification in the *M. paratuberculosis* 19 kDa protein. Following the analysis of the expressed 19 kDa by deglycosylation enzymatic assay and conA staining, there was clear evidence that the modification of this protein is not due to glycosylation (Figures 19 and 20). We strongly conclude that unlike the glycosylation of *M. bovis* 19kDa protein, the 19k, antigen in *M. tuberculosis* and the 45-k protein in *M. tuberculosis* (19, 20, 13, 12, 7), the 19 kDa protein of *M. paratuberculosis* is not a glycoprotein and may be subjected to other posttranslational modification mechanism

Finally, we further investigated the role, if any, of posttranslational modification component of the expressed 19 kDa *M. paratuberculosis* in the protein antigenicity and proliferative capability. Specifically, our concern focused on whether the unexpressed portion of the protein (16 kDa in *E. coli*, Figure 3) may encode immunomodulatory components that are essential in the exposure of the 19 kDa antigen during natural infection in cattle and humans. Initially, both forms of the expressed protein (16 kDa in *E. coli* and 19 kDa in *M. paratuberculosis*) reacted similarly to rabbit-anti-*M. paratuberculosis* IgG antibodies (Figure 3). Most recently, we evaluated both forms of the 19 kDa protein against T-lymphocytes derived from two healthy individuals and two patients with Crohn's disease. T-cell response to PHA, a non-selective mitogen, has shown to be less in Crohn's disease compared to healthy controls. This finding confirms earlier observation in our lab (Romero, submitted). Preliminary data so far suggest a

minor decrease in the proliferative response of T-cells reactivity to protein extracts containing the 16 kDa compared to the 19 kDa forms (Table 4). This suggests that the 3 kDa component of the 19 kDa protein may not play a role in the immunogenicity of the protein.

CHAPTER FIVE CONCLUSION

Collectively, our data show data clearly illustrate for the first time that *M. paratuberculosis* supports posttranslational modification. The data confirms observation reported in *M. tuberculosis*, *M. bovis* and *M. leprae*. The significance of this finding may aid in investigating virulence in *M. paratuberculosis* and its pathogenesis in patients with Crohn's disease and animals with Johne's disease. Our data also confirm that the posttranslational modification of this 19 kDa protein from *M. paratuberculosis* is not due to Glycosylation. The presence of series of serine in the peptide sequence of the 19 kDa may suggest that acylation should be investigated. The role of the posttranslational modification component seems to be limited since there was limited difference between the two forms of the protein (16/19 kDa) against T- cell response.

APPENDIX A

FIGURES

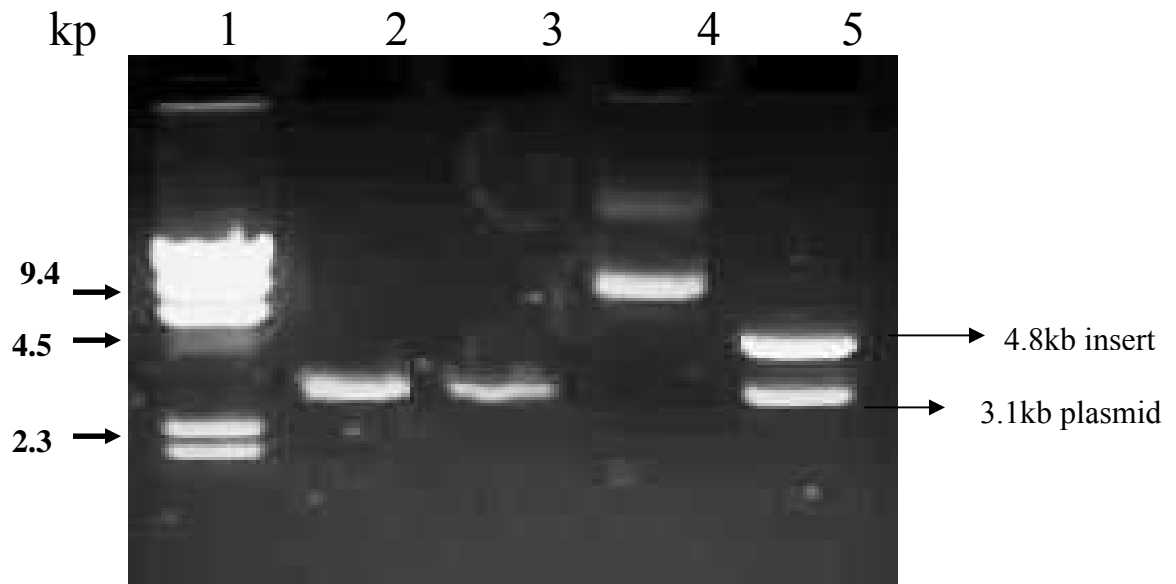


Figure 1: Isolation and Restriction Endonuclease Digestion of Extracted Plasmid from Clone pMptb#28

Plasmid pMptb#28 and vector pcDNAII were extracted and digested by enzyme *Bam* HI.

Both digested and undigested plasmids were analyzed on 0.8% agarose gel.

Lane1, DNA/*Hind* III size standard, Lane2 and 3, Plasmid pcDNAII circular and

linearized, Lane3 and 4, Plasmid pMptb#28 undigested and digested

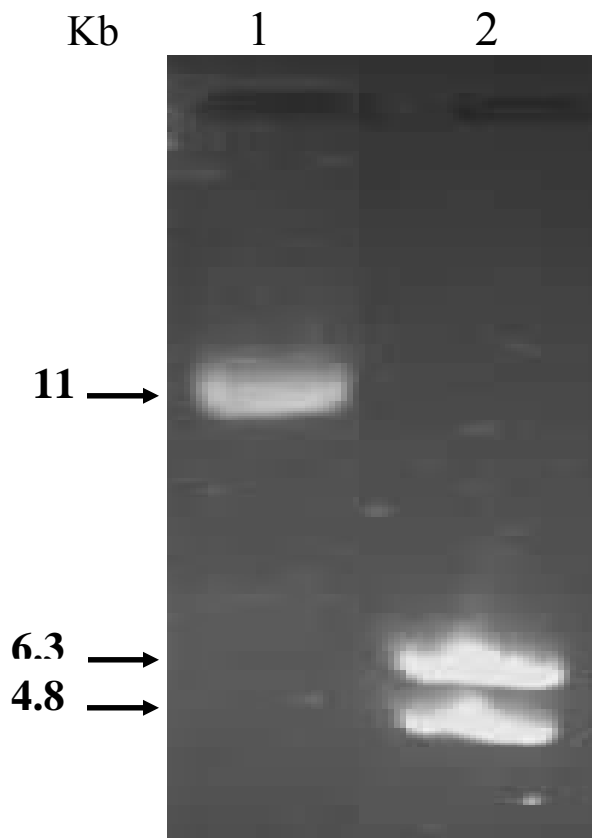


Figure 2: Restriction Endonuclease Digestion of Extracted Plasmid from Clone Smeg19k

Plasmid extraction from recombinant clone Smeg19k was performed and then treated by restriction endonuclease digestion *Bam*H I.

Lane 1, undigested plasmid Smeg19k, lane 2, digested plasmid Smeg19k representing 6.3kb vector and 4.8kb insertion fragment.

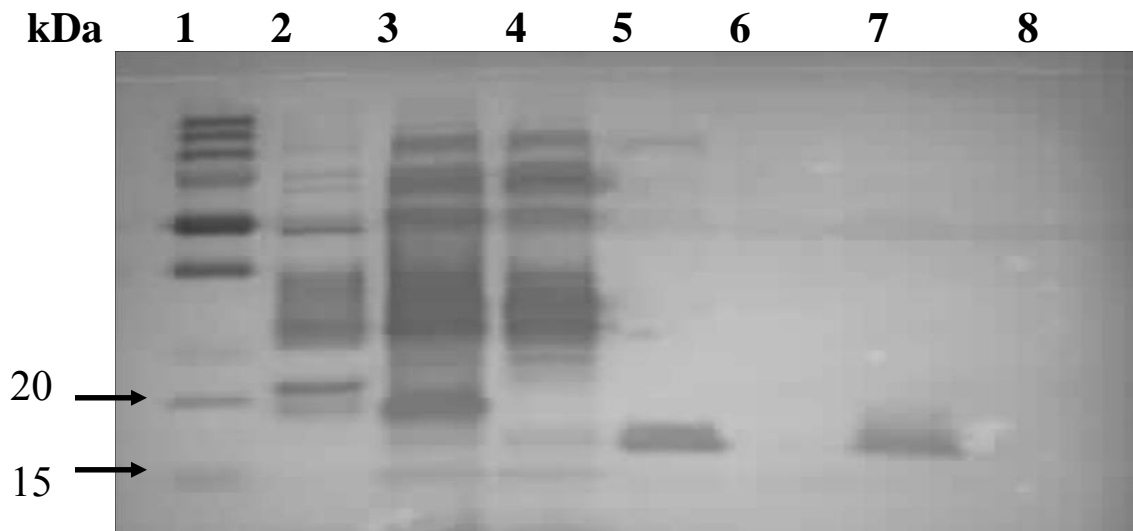


Figure 3: Immunoblot Analysis of the Native 19kDa Protein (*M. paratuberculosis*) and the Recombinant Proteins (*E. coli*, *M. smegmatis*)

Protein extracts of *M. paratuberculosis*, *M. smegmatis* recombinants, *E. coli* recombinants were loaded on SDS-PAGE and immunoblotted against *E. coli* adsorbed rabbit anti-*M. paratuberculosis* sera.

Lanes: 1, protein size standard, Lane2, extracts from *M. paratuberculosis*, Lane 3, 4, extracts from smeg/shuttle19 and Smeg/shuttle Lane5, 6 extracts from *E. coli*Mptb#28 and *E.coli* pcDNAII, Lane7, 8 extracts from *E. coli*/shuttle19 and *E. coli*/shuttle

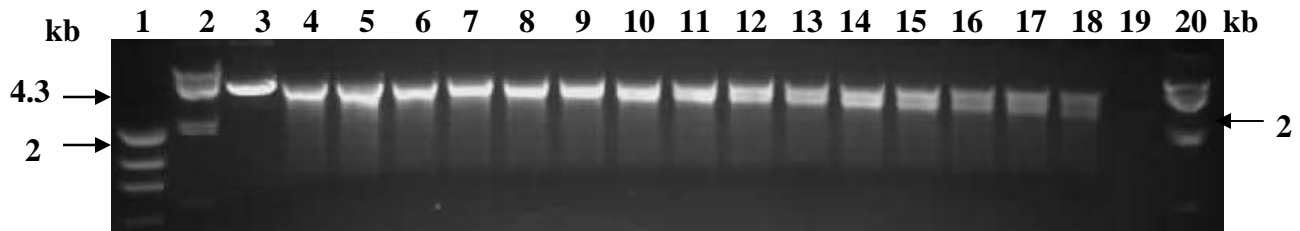


Figure 4: Identification of DNA nested deletion of pMptb#28

The extracted plasmid of pMptb#28 was digested first by *XhoI* and then *SpeI*. The created 5' overhang close to insertion was susceptible to ExonucleaseIII activity. The DNA sample was exposed to ExonucleaseIII, aliquots of deleted DNA were removed every 30 sec and then the reaction was stopped with S1 stop mixture. Deleted DNA aliquots were analyzed by gel agarose electrophoresis.

Lane 1: Low mass DNA standards Lane 2 & 20: λ Hind III size standards Lane 3 to 18 contain DNA deletion aliquots removed from 0 sec to 10 min.

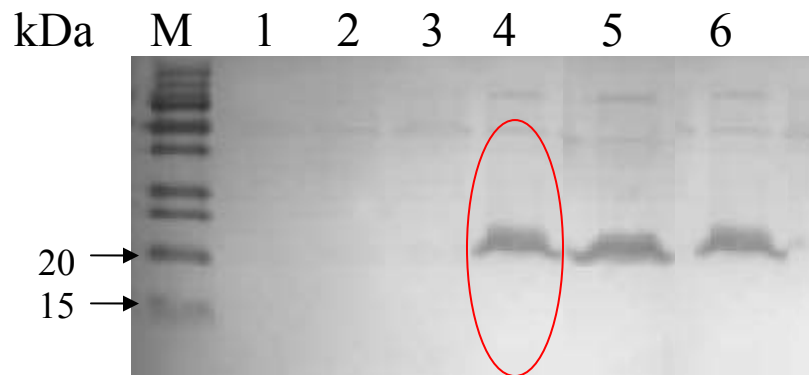


Figure 5: Immunoblot analysis to identify the protein expression from DNA nested deleted subclones

The time deleted plasmids were selected to re-circularize, then transformed to *E. coli* Top 10. The subclones were examined at protein level by immunoblot using rabbit anti-*M. paratuberculosis* antibody to reveal the smallest plasmid that is still able to produce the protein.

Lanes: M, protein size standard, Lane 1, 2, 3, 4, 5, 6 are extracts proteins from subclones

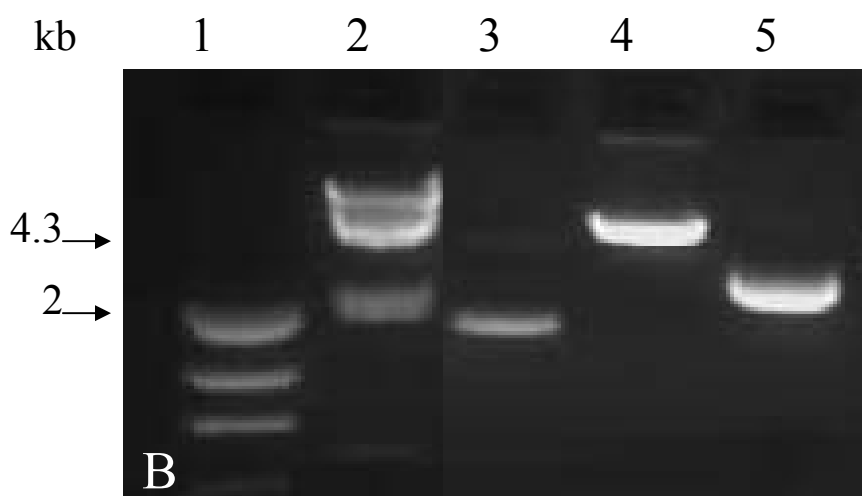
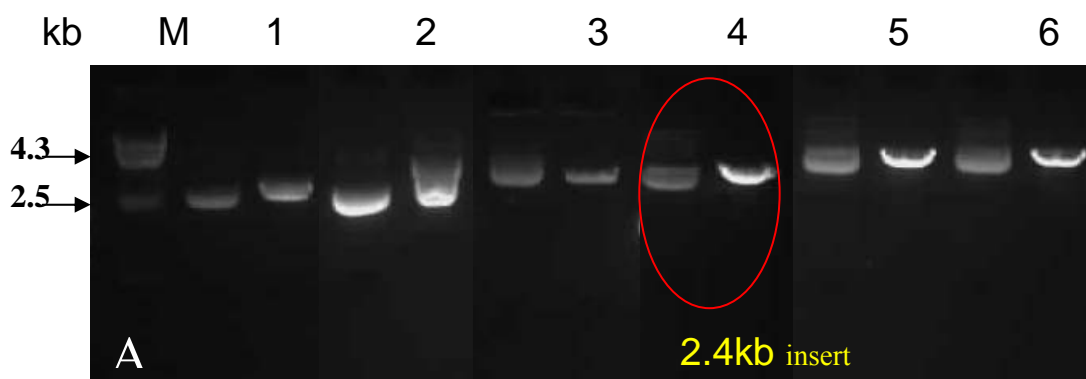


Figure 6: Plasmid Extraction and Digestion of DNA Nested Deleted Subclones

Plasmid extraction were carried on some of the DNA nested deleted subclones and then linearized by restriction endonuclease *kpn* I. Circular and linearized plasmids from subclones, pcDNAII and pMptb#28 analyzed on 1% agarose gel.

Panel A: Lane M, low mass DNA ladder size standard, Lane 1-6, circular and linearized plasmids from subclone 1,2,3,4,5,6

Panel B: Lane 1,2, low mass DNA ladder and DNA/*Hind* III size standard, Lane 3, linearized pcDNAII vector, Lane 4, linearized pMptb#28 plasmid, Lane5, linearized plasmid clone #4(the smallest plasmid, which still producing protein.

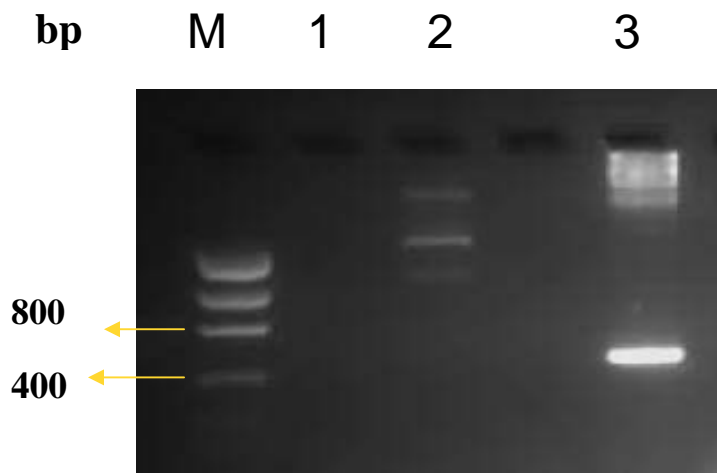


Figure 7: PCR Analysis of 486bp ORF Region

Polymerase Chain Reaction was carried using the primers to amplify the ORF from 4.8 kb fragment in clone pMptb#28, then analyzed on 2% agarose gel.

Lane M, low mass DNA ladder standard size, Lane 2, PCR negative control, Lane 2, second negative control when pcDNAII used as template, Lane3, PCR product 506 bp including 486 ORF and added restriction enzyme sites.

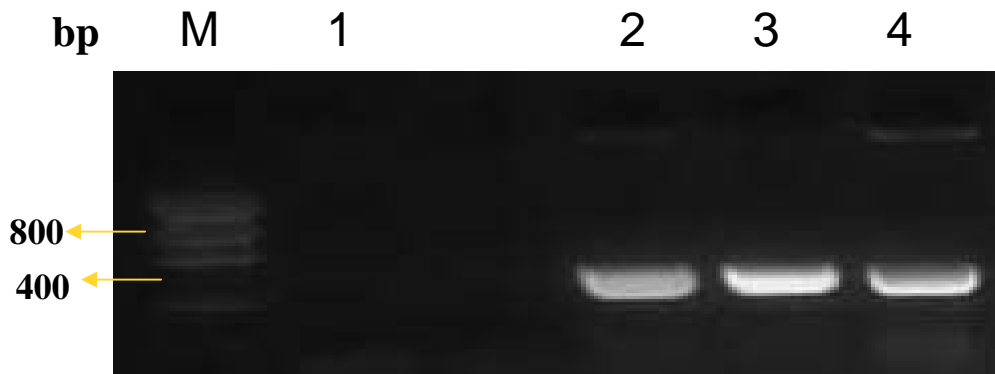


Figure 8: Cell Pop PCR Technique to Screen Subclones

Subclones were randomly picked as template to perform PCR using the primers. This study was carried to identify the presence of insert (486bp ORF) without plasmid extraction.

Lane M, low mass DNA ladder standard size, Lane 1, PCR negative control, Lane2,3,4, PCR product of three different subclones randomly picked from the plates.

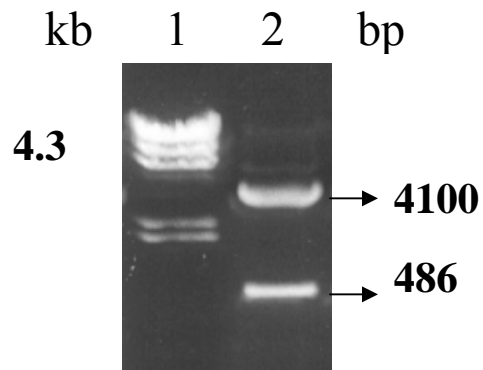


Figure 9: Restriction Endonuclease Digestion of Extracted Plasmid from Subclone EB486

Plasmid extraction was performed on one of the screened colony by Cell Pop PCR, and digested by Bgl II and EcoR I to analyze on 1% agarose gel.

Lane 1, DNA/*Hind* III size standard, Lane 2, Digested plasmid representing 4.1kb pBAD vector and 486bp ORF fragment.

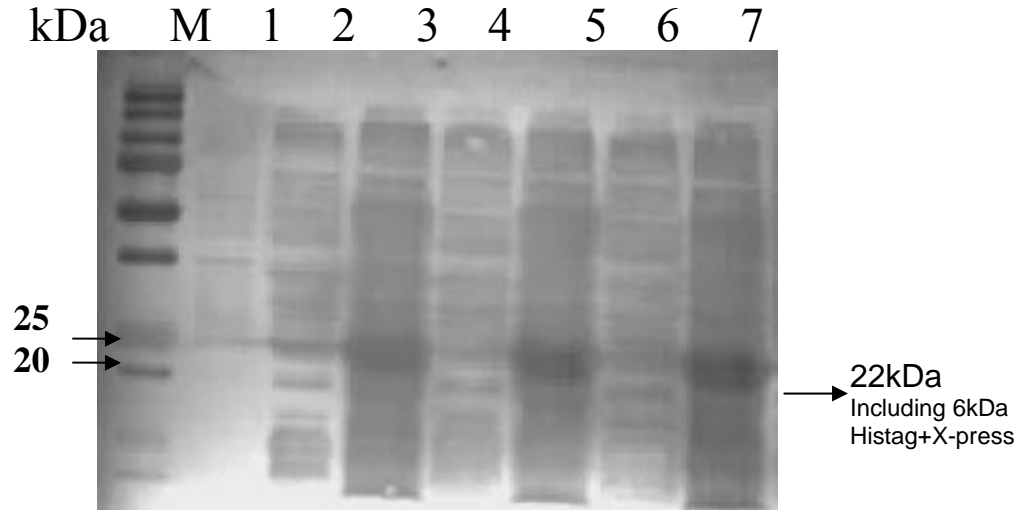


Figure 10: Immunoblot Analysis of Subclones (EB486) By Anti-His Antibody

Positive subclones by Cell Pop PCR were grown in LB and induced by L-arabinose.

Then, cell lysates (induced and un-induced) were fractionated on SDS-PAGE gel and transferred on to nitrocellulose membrane. Anti-His antibody was applied to identify the fusion protein.

Lanes: M, protein size standard, Lane 1, Negative control, cell lysate from pBAD, Lane

2,3 cell lysate clone #1(un-induced and induced), Lane 4,5 cell lysate

clone#2,(uninduced and induced), Lane 6,7 cell lysate clone#3(uninduced and induced),

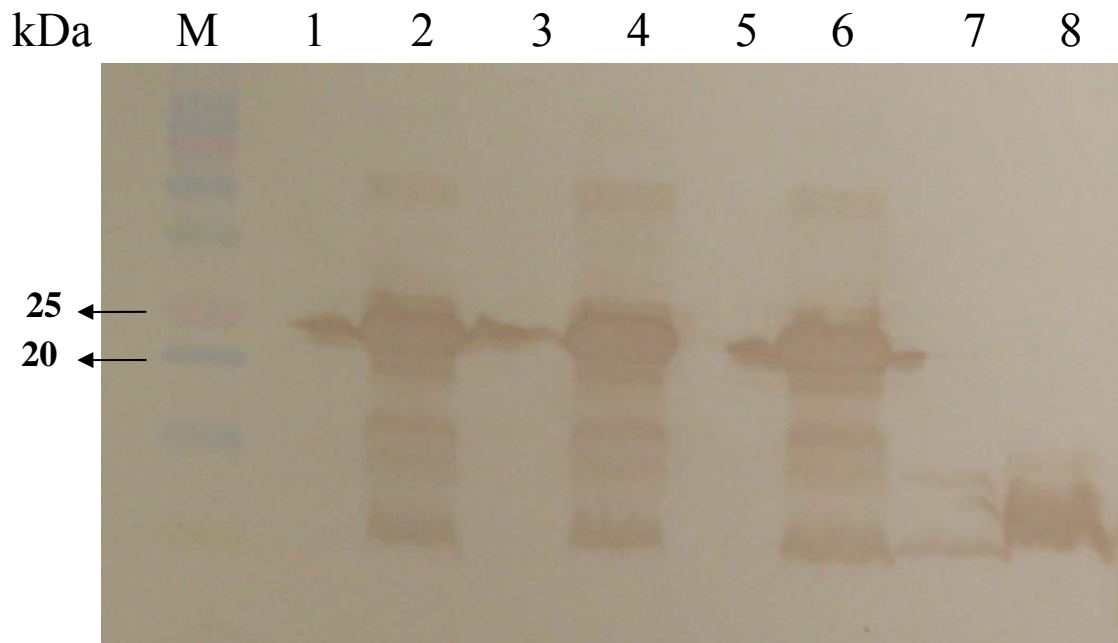


Figure 11: Immunoblot Analysis of Subclones (EB486) By Anti-Xpress Antibody

Positive subclones by Cell Pop PCR were grown in LB and induced by L-arabinose.

Then, cell lysates (induced and un-induced) were fractionated on SDS-PAGE gel and transformed on to nitrocellulose membrane. Anti-Xpress antibody was used to identify the fusion protein.

Lanes: M, protein size standard, Lane 1,2 cell lysate clone #1, un-induced and induced, Lane 3,4 cell lysate clone#2,un-induced and induced, Lane 5,6 cell lysate clone#3, Lane 7,8 Negative control, cell lysate from pBAD, un-induced and induced.

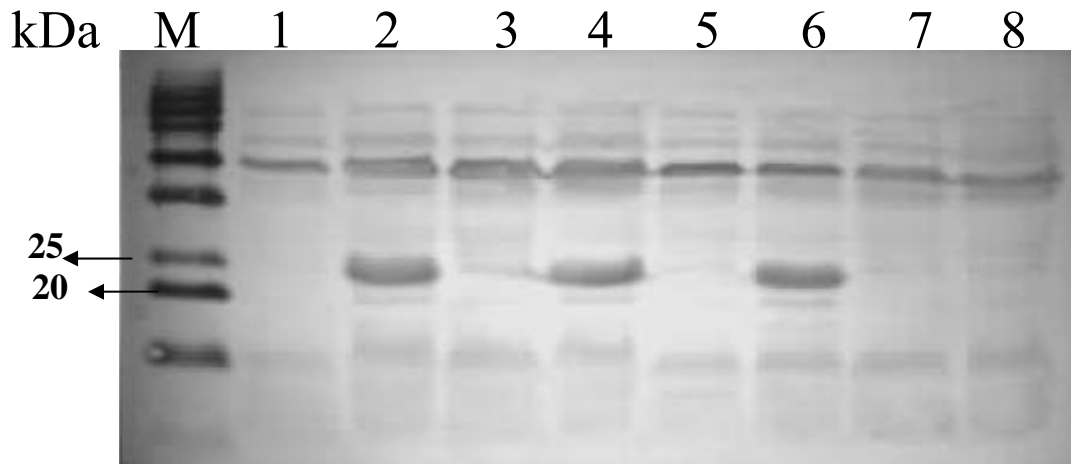


Figure 12: Immunoblot Analysis of Subclones (EB486) By Rabbit Anti-MAP Serum

Positive subclones by Cell Pop PCR were grown in LB and induced by L-arabinose.

Then, cell lysates (induced and un-induced) were fractionated on SDS-PAGE gel and transferred on to nitrocellulose membrane. *E. coli* adsorbed rabbit anti-*M. paratuberculosis* sera was used to identify the fusion protein.

Lanes: M, protein size standard, Lane 1,2 cell lysate clone #1, un-induced and induced,

Lane 3,4 cell lysate clone#2,un-induced and induced, Lane 5,6 cell lysate clone#3, Lane

7,8 Negative control, cell lysate from pBAD, un-induced and induced.

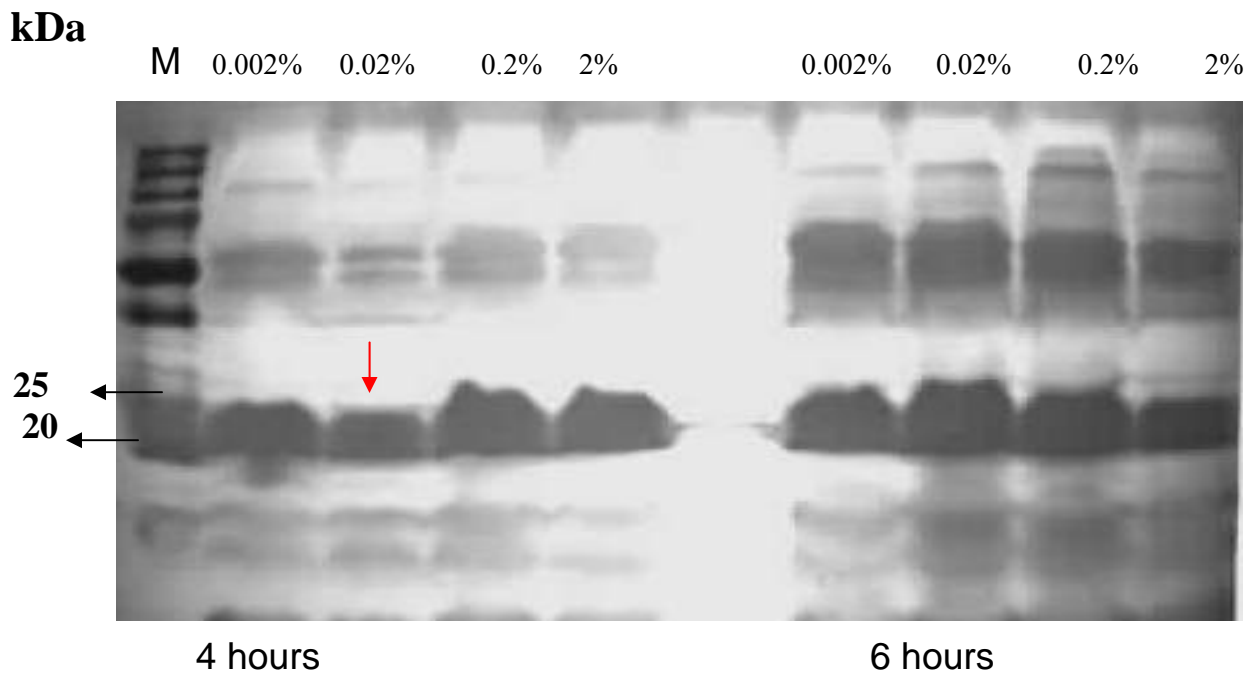


Figure 13: Optimization of Recombinant Protein EB486 Expression

Different concentration of L-arabinose (0.002%, 0.02%, 0.2%, and 2%) was induced in different time incubation of 0hr, 2hr, 4hr, 6hr, 8hr, and overnight (picture is showing just 4 and 6 hr) to determine optimal condition for expressing the EB486 protein. Cell extracts were fractionated on SDS-PAGE and blotted on a nitrocellulose membrane, which screened by Anti-His antibody.

Lanes: M, An arrow on picture is showing the best condition, 0.02% concentration of L-arabinose and 4 hr incubation time after induction

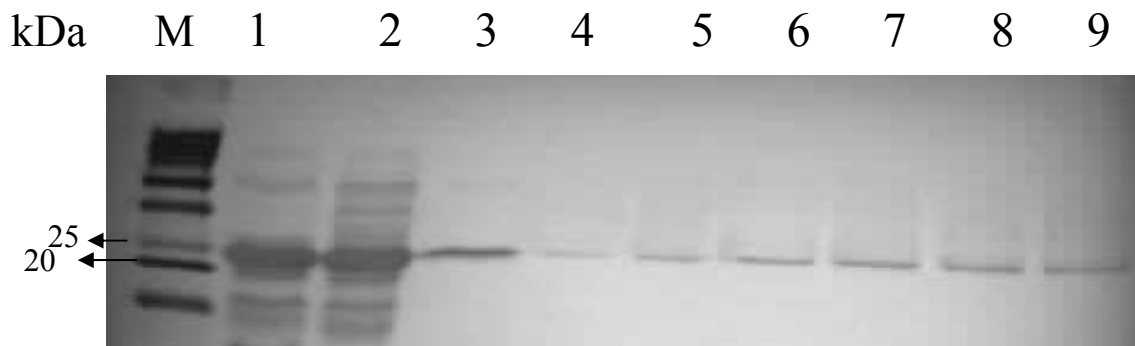


Figure 14: Purification of Histidine-tagged EB486 Recombinant Protein

Recombinant EB486 protein was expressed under the optimal condition. Then cell pellet from 200ml culture were purified using the PIERCE kit. Fractions were collected from each of the purification steps and assayed by SDS-PAGE. Then Immunoblot was performed using anti-His antibody.

Lane M, protein size standard, Lane 1, cell crud lysate Lane 2, flow-through, Lane 3, 4,5 three washes Lane 6,7,8,9 three Elutes

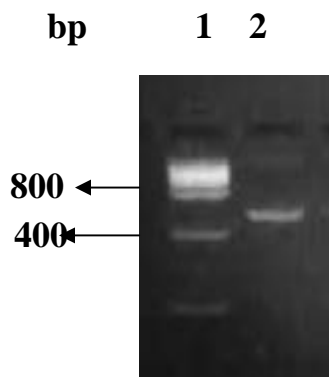


Figure 15: PCR Analysis of 486bp ORF Region for Cloning into *M. smegmatis*

Polymerase Chain Reaction was carried using the primers to amplify the ORF from 4.8kb fragment in clone pMptb#28, then analyzed on 2% agarose gel.

Lane 1, low mass DNA ladder standard size, Lane 2, PCR product 506 bp including 486 ORF and added restriction enzyme sites (*Bam*H I and *kpn* I

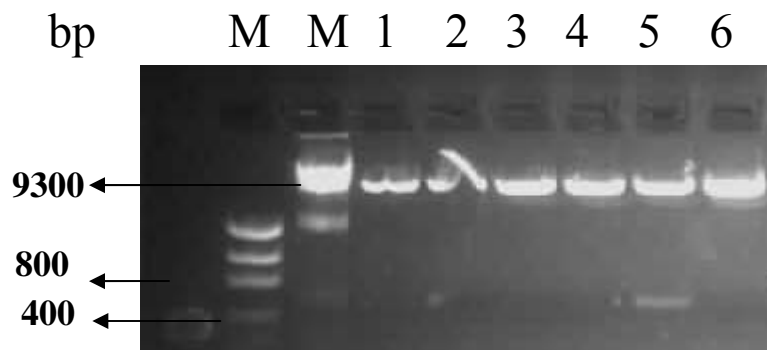


Figure 16: Restriction Endonuclease Digestion of Extracted Plasmid from *E. coli* Subclone (Transition)

Plasmid extraction was performed on one of the screened colony by Cell Pop PCR, and digested by *Bam*H I and *kpn* I to analyze on 1% agarose gel.

Lane M, Low mass DNA ladder and DNA/*Hind* III size standard, Lane 1-6, Digested extracted plasmid from transformed colonies, Lane #5 digested plasmid representing 6.8kb shuttle vector and 486bp ORF fragment.

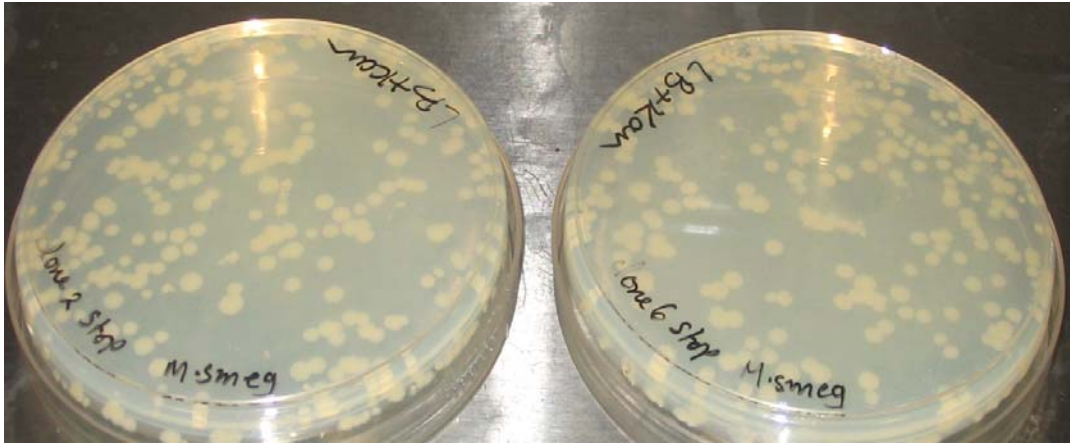


Figure 17: Transformation of Shuttle vector to *M. smegmatis* strain mc2155

Shuttle vector including 486 bp ORF from *E. coli* Subclone was electroporated to *M. smegmatis* mc2 155.

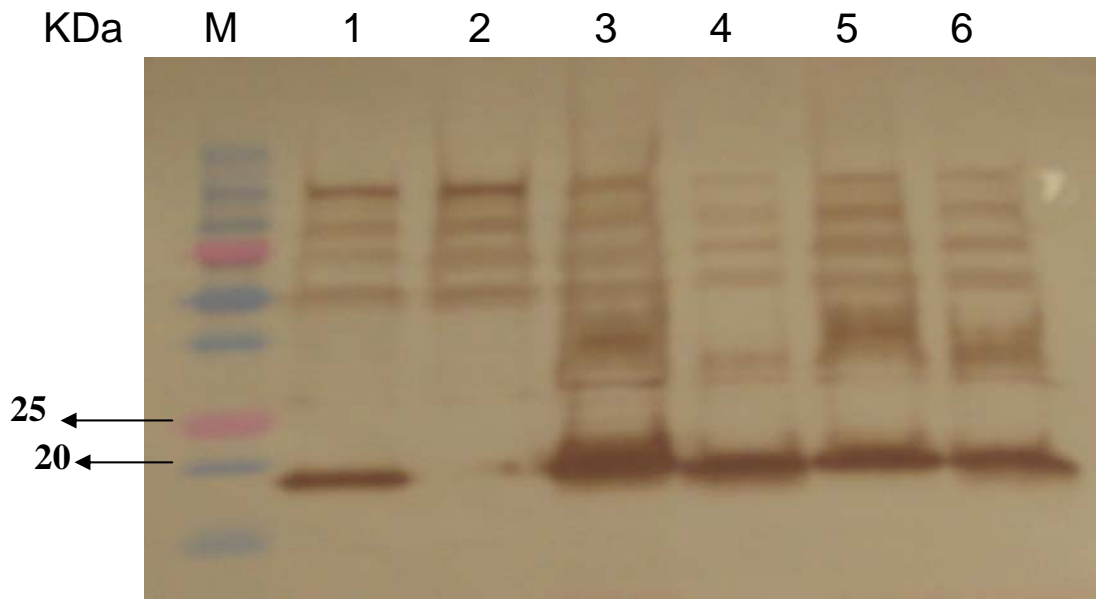


Figure 18: Immunoblot Analysis of *M. smegmatis* Subclones(SM486)

M. smegmatis subclones were grown in 7H10 and then sonicated. Protein extracts of *M. smegmatis* recombinants were fractionated on SDS-PAGE gel and immunoblotted against *M. smegmatis* adsorbed rabbit anti-*M. paratuberculosis* sera.

Lanes: M, protein size standard, Lane1 extracts from smeg/shuttle19(including 4.8 kb fragment), Lane 2, Smeg/shuttle as negative control, Lane3,4,5,6 *M. smegmatis* subclons (SM468)

GATCCTGGGCTCGATCGGTGCGCCGGTGCCTGCGCCGGCGCTACTGGTACTGACCGGCCGGCGGTTTCACAGGCCACGCAAA
GGTAGGGCATAGCCGACGCTCAGCCCGCAGCGGATACTGGGACATGTGAGCCAGCCGCGAGCCTCAGCCGAGCGGGTCTGTC
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TGGCTACGAGGGTGAACATCGCCACCCGACGCGAGCGGCTCGGCCATGCGCGCCGCGCAACCAAGCCGCGGACG
TGGTGGTCTCGACGTGATGCTGCCCCGACATGAGCGGGCTGGACGTGCTGCAACAAGCTGCGGGAGGAGAACCCCCAGCTGCC
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CACCGAGTTCGAGCTGTTGCGGTTTATGATGCGCAACTCCAAGCGGGTGTGAGCAAGGCTCAGATCCTGGACCGGGTGTGG
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GGTGGTGCAGCCACAACGTCGAAGGGATCTACCAGCGCTACATGGGCTGGTTCGACGGCAACCCGGCCCGGCTGTGGCCCC
ATCCCCCGAGGGCGTGGGGCCCCGCTATGTGGTTCGATGGGCGGATCGACCGGGTCTGACCTCGCCAGCCAGCGGCGT
TCGACTCCGGTATTACCGTTGGGAGCAACCCCTTTGGACACCGGATGTTACCCGACGGTGGACGCGCGCGGCGCCGCGA
GCTCTACGCCGACGCTCGAGCAGCTGGCCACGGCGAGAGAACGCGACCTGGCGCAACTTCTTCTCAGCGGGGCAACG
GAGCTGCGCGACGGCAATTTCCGCAACCGCCGGGCAAGGTACCTCGCCACCATGCTTGCACGCTGACGCGGAGCAGATCT
TCGACGGCCTGGCCATCCGGTCAACGGGCCACGACGCTGGGGCCTCGACATACCCGTCGATGTTACCTTGGCGACAGCG
GGTCAACCATCGGCTCGCGCAACG GCGTGTGTTGCAACGCAAGGTGCGCGC

Figure 19: Nucleotide Sequence analysis of the 4778bp including 486bp encoding the 19kDa protein

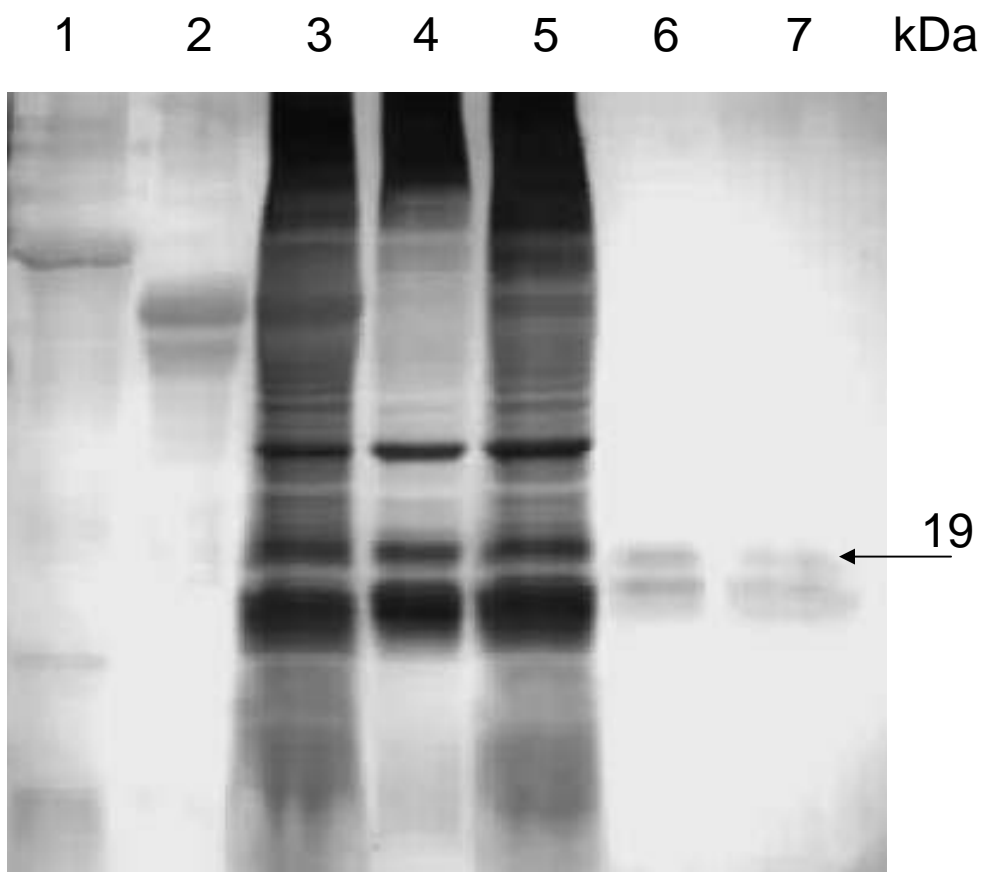


Figure 20: Carbohydrate Detection: Effect of Deglycosylation on Mobility

Silver Staining of samples before and after deglycosylation

Bovine Fetuin as positive control and cell crude lysate from recombinant clone

smeg/pShuttle 19 loaded in 12%SDS gel before and after applying deglycosylation kit.

Lanes: 1,2 Bovine Fetuin before and after deglycosylation 3, 4 cell lysate from

recombinant clone smeg/shuttle19 before and after deglycosylation 5, spike (mix of

positive control and cell lysate smeg/shuttle19) before and after deglycosylation 6,7,

purified 19kDa recombinant protein from smeg/shuttle19k (eluted from polyacrylamide

gel) before and after deglycosylation.

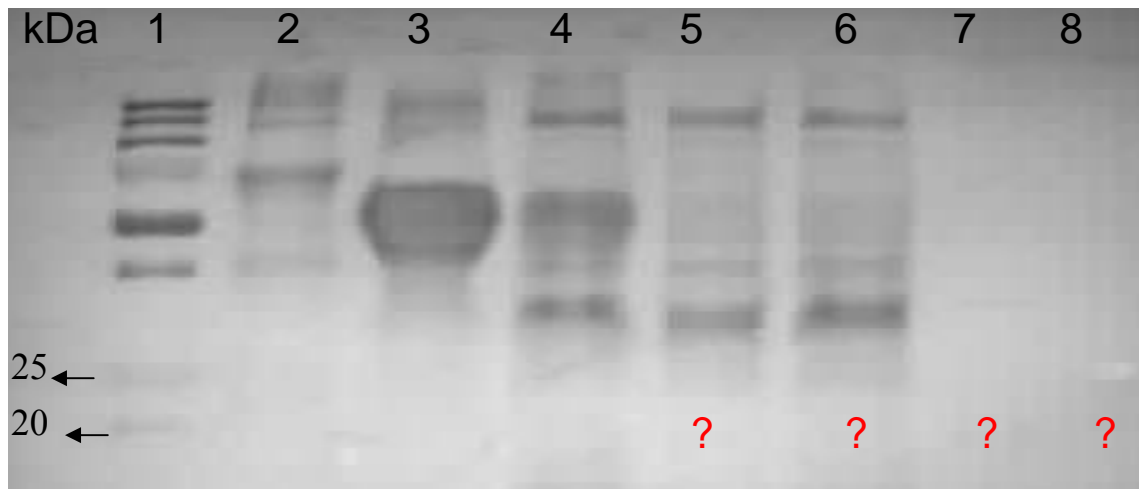


Figure 21: Carbohydrate Detection: Concanavalin-A staining

The same samples used in figure 7 were analyzed by SDS-PAGE and followed staining with peroxidase-conjugated ConA. As shown ConA was positive for Bovine fetuin (positive control) but not in samples from smeg/shuttle19k in expected size. This confirms the lack of glycosylation in recombinant protein produced by *M. smegmatis*.
 Lanes: 1, protein size standard, 2,3 Bovine Fetuin before and after deglycosylation , 4,5 cell lysate from recombinant clone smeg/shuttle19 before and after deglycosylation 6, spike (mix of positive control and cell lysate smeg/shuttle19) before and after deglycosylation, 7,8, purified 19kDa recombinant protein from smeg/shuttle19k(eluted from polyacrylamide gel) before and after Deglycosylation

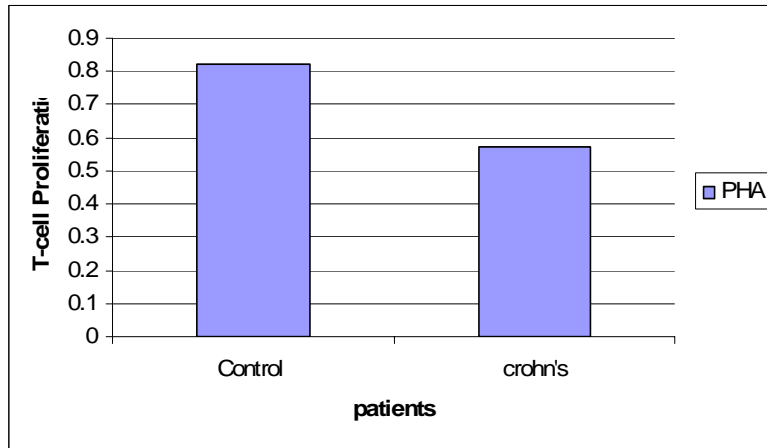


Figure 22: T-Cells proliferation response in Healthy controls compare to Crohn's diseases

APPENDIX B

TABLES

Table 3: Evaluation of PBMC Proliferative Response to PPD and Positive Controls

	PWM(b)	PHA(a)	PPD(c) (PPD \leq 10%Environmental exposure)
Healthy control1	Normal	Normal	11%
Healthy control2	Normal	Normal	10%
Healthy control3	Normal	Normal	2.5%
Crohn's patient1	Normal	Suppressed (40%)	16%
Crohn's patient2	Normal	Suppressed (67%)	6.5% (map exposure)

a-PHA: Phytohemagglutinin to assess the overall function of lymphocyte responsiveness

b-PWM: Pokeweed mitogen to assess B cell responsiveness dependent on T-cell activation

c-MAP PPD: Purified protein derivative from MAP to assess previous subject's exposure to the bacterium

Table 4: Evaluation of PBMC Proliferative Response to Various Proteins

	PHA	Extract Smegw/insert	Extract Smeg	Extract <i>Ecoli</i> w /insert	Extract <i>Ecoli</i>	Purified 19kDa	Purified 16kDa
Healthy control1	0.76	0.25	0.171	0.2	0.185	N/A	N/A
Healthy control2	0.8	0.265	0.213	0.232	0.22	0.29	0.274
Healthy control3	0.9	0.125	0.11	0.198	0.177	N/A	N/A
Crohn's patient1	0.53	0.281	0.167	0.25	0.158	N/A	N/A
Crohn's patient2	0.37	0.261	0.22	0.153	0.142	0.265	0.22

PHA: Phytohemagglutinin to assess the overall function of lymphocyte responsiveness

N/A: not enough available blood sample or protein available

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