# MEMBRANE SERINE PROTEASE PROTECTS *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* AGAINST PHAGOSOMAL ACID STRESS

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## Abstract

Pathogenic mycobacteria survive in the acidic environment of the phagosome. We hypothesize that a serine protease of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), encoded by MAP0403, aids in the resistance to phagosomal acidification and is critical for survival in macrophages. The modulation of expression of MAP0403 within macrophages by MAP K-10 was studied. Bafilomycin treatment was used to block phagosomal acidification. Gene encoding the MAP serine protease was significantly up regulated in the acidified phagosomes. Highest levels of MAP0403 expression correlated with peak phagosome acidification in macrophages. Bioinformatically predicted genes that encode proteins interacting or are co-expressed with MAP0403 during phagosomal acidification were analyzed by microarray. Results show that the genes involved in DNA repair, protein synthesis and those located immediately up stream of MAP serine protease are up regulated in the acidified phagosome. Inasmuch as *Mycobacterium smegmatis* cannot resist and persist in the acidified phagosome, we cloned the open reading frame of MAP0403 into *M. smegmatis* $mc^2$ *155*. Compared with controls, *M. smegmatis* $mc^2$ *155* transformants carrying the MAP serine protease show increased survival during in *vitro* acid stress and in monocyte derived macrophages. Further, we show that serine protease carrying *M. smegmatis* transformants are able to maintain intra-bacterial pH when exposed to an acidic media (pH~5), while controls failed to do so. Our studies suggest that MAP serine protease is critical in resisting the phagosomal acidification by MAP.

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# Chapter 1

## Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) survives in host macrophages amid severe oxidative stress and nutrient deprivation. Phagosomal acidification is a major host mechanism involved in destroying phagocytized bacteria via various mechanisms such as activation of lysosomal hydrolases to increase proteolytic degradation of the bacteria, enhance concentration dependant hydrogen ion influx into bacteria by maintaining high concentration of hydrogen ions in the phagosomal compartment. Intra bacterial pH homeostasis is vital for cellular biological processes and stability of nucleic acids and proteins in pathogenic mycobacteria. Basic mechanism by which mycobacteria regulate intra-bacterial pH is not completely understood. A membrane associated serine protease of *Mycobacterium tuberculosis* (MTB) has been shown to play a key role in maintaining the intra-bacterial pH [1]. A recently described membrane associated serine protease of MTB is conserved across mycobacteria with 94% amino acid identities between *Mycobacterium tuberculosis* and MAP.

We hypothesized that serine protease of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), encoded by MAP0403, aids in the resistance to phagosomal acidification and is critical for survival in macrophages. We tested this hypothesis under 3 aims.

**Aim 1:**

Determine the in-vitro pH modulation of expression of MAP0403, a serine protease, in pathogenic and non-pathogenic mycobacteria

**Aim 2:**

Study the expression of MAP0403 by intracellular MAP in monocyte derived macrophages and epithelial cells.

**Aim 3:**

Demonstrate the importance of MAP serine protease in resisting acid stress, using non-pathogenic *M. smegmatis mc$^2$155* as a model system.

# Chapter 2

## Literature Review

### Johne's disease

Johne's disease (JD) is a worldwide problem of domestic and wild animals and of potential public health concern. A chronic granulomatous inflammatory intestinal disease, JD results from infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Since its first description over a century ago, JD is now recognized to be a serious economic and animal health problem in domesticated ruminants (such as dairy and beef cattle, sheep, and goats) throughout the world, resulting in more than $200 million in annual losses to US agriculture each year. The growing recognition of MAP infection in wildlife species is also of considerable concern. Similarly, recent evidence of the presence of MAP in retail milk sources is of concern from a milk quality and potential food safety standpoint.

A report from the National Research Council of the US National Academies of Sciences on JD comprehensively reviewed the literature, identified major gaps in knowledge, and provided clear recommendations for future research priorities and strategies for the prevention and control of JD.[2] In brief, the report concluded that JD is a significant animal-health problem whose study and control deserves high priority from the USDA. It was recognized that the problems associated with JD stem from: (i) difficulties in diagnosis because of an unusually long incubation period and a lack of specific and sensitive diagnostic tests for detecting early infections; (ii) a lack of vaccines or other effective measures for infection control; and (iii) a lack of general awareness of the disease and its true economic and animal-health consequences. The report made 25

specific recommendations regarding implementation of strategies for the control of JD, education and training of producers and veterinarians and filling of key gaps in knowledge relating to JD. A key missing element in JD research is a comprehensive understanding of MAP-macrophage interactions that are critical to establishment of MAP infection.

### *Mycobacterium avium* subspecies *paratuberculosis*

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a facultative intracellular, acid-fast positive rod that belongs to genus *Mycobacterium*. It is a slow growing pathogen that takes 6 to 8 weeks to grow under laboratory conditions with a generation time of 20h [3]. Species specific IS*900* insertion sequence polymerase chain reaction is used to identify MAP. Both MAP-K10 and MAP 1018 have been characterized by short sequence repeats (SSR) typing by the two polymorphic loci (G and GGT repeats) and by microarray analysis for genome content differences. MAP-K10 (15G, 5GGT) is a virulent, low passage clinical isolate from a Wisconsin dairy herd. The genome sequence was closed in 2005 [4] and re-annotated recently [5]. This strain is also phenotypically well characterized [6-10]. Thus, MAP K10 is used as a classical strain to test the hypothesis in most of the studies [6, 8]. MAP 1018(7G, 4GGT) is a clinical isolate from Ohio dairy herd. MAP 1018 is also been genotypically and phenotypically well characterized [6, 7, 11]. MAP 1018 is prevalent in many parts of the world and MAP K-10 is a predominantly spread across the US. MAP 1018 was used to test the hypothesis due to the availability of genomic, proteomic and transcriptomic details and the worldwide distribution.

**Mycobacteria and phagosome**

MAP is transmitted via fecal-oral route. When MAP reaches the intestine, it internalized into the intestinal epithelium or into microfold cells (M cells). Internalization into epithelial cells is a multifaceted process that is mediated through fibronectin, mannose, and complement receptors and the major membrane protein of MAP [12, 13]. Fibronectin mediated phagocytosis is achieved via a bridge between fibronectin-FAP-P complexes and the β1 integrins located on the intestinal epithelial cells [13, 14]. Subsequently, MAP orchestrates its exit from epithelial cells into submucosal macrophages or dendritic cells where an intracellular niche is established. Macrophages have mannose receptors and complement receptors that bind with MAP and enhance phagocytosis. In order to survive the hostile intracellular environment; the phagocytized pathogen has to escape from the phagosome to cytosol; prevent the phagosome from acidification; or develop the ability to survive in the low-pH environment of the acidified phagosome.

The host cells deploy three major types of assault on MAP: Oxidative stress, acid stress, and nutritional deprivation, while it resides in the phagolysosome. Pathogenic mycobacteria respond by resisting and persisting in the acidic environment of the phagosome or phagolysosome without escaping to the cytosol: modifying the phagosome, resulting in inhibition of acidification, phagosome-lysosome fusion and lysosomal enzyme activities; resist or neutralize the damaging effects caused by reactive intermediates and suppress the macrophages responsiveness to activating cytokines such as gamma interferon and  interleukin 10 [15-22]

Despite the mycobacterial responses, lysosome fuse with MAP containing phagosome in about 10-30 minutes post infection. Lysosomal proteases are most active at acidic pH. Vacoular ATPases (V-ATPases) that are located on the lysosomal membrane pump $H^+$ ions into the phagosome and leading to a drop in pH from 6.8 to about 5 [23, 24]. This event occurs in about in 10 minutes post phagocytosis. Acidity of the phagolysososme equilibrates in 15-60 minutes [23, 24].

Maintenance of intra-cytoplasmic pH is essential for all bacteria [25]. A change in the intra-cytoplasmic pH is detrimental to all biological process. Reduced intra-bacterial pH alters enzyme activity and reaction rates, protein stability, structure of nucleic acids and other biological processes. However, some bacterial families have evolved specialized mechanisms to maintain a narrow range intra-bacterial pH. Environmental mycobacteria are exposed to acidic environment in their natural soil/water habitat, while pathogenic mycobacteria encounter an acidic environment inside phagocytic cells. *M. tuberculosis* and MAP are able to “perceive” the pH of their environment, respond and survive in an acidic environment .

Nascent phagosomes acquire microbicidal properties upon the sequential fusion events with various endocytic vesicles into phagolysosomes. Phagolysosomes are acidic organelles with hydrolytic enzymes that kill and digest engulfed foreign bodies including bacteria. Pathogenic mycobacteria are neutrolophiles in that they maintain a near neutral intra-bacterial pH even when exposed to acidic environment [27, 29]. Intra-cytoplasmic pH is maintained by influx or efflux of protons across the membrane. Intra cellular pH

homeostasis can be either active or passive process. Passive cytoplasmic buffering greatly contributes to maintain the cytoplasmic pH while the active mechanism occurs when the bacterium needs to rapidly adjust the intra-cytoplasmic pH in response to the exposure to a higher magnitude of pH change. Permeability of bacterial cytoplasmic membrane to protons plays an important role in maintaining a near neutral intra-cytoplasmic pH [30]. Adjusting to extreme extracellular or intracellular changes is an orchestrated process modulated via several metabolic pathways [11, 31-33].

**Trypsin like serine proteases**

Protease is an enzyme that hydrolyses the peptide bond, a covalent bond that is formed between the carboxyl group of one amino acid and the amino group of the other amino acid of the protein. The reaction is irreversible and it modifies the function of the substrate protein. Proteolytic activity can activate, inactivate or alter substrate-specificity, and ultimately modify signaling circuits and cell function. Trypsin-like serine proteases are the largest group of proteolytic enzymes conserved across both prokaryotes and eukaryotes. Trypsin like serine proteases have a unique catalytic triad made of aspartate, histidine and serine where the nucleophilic serine residue initiates the enzymatic reaction [34].

Forty-three ORFs in the MAP genome are either established or predicted as proteases. Thirty-eight of the 43 putative proteases are conserved among *M. tuberculosis, M. leprae* and *M. bovis* [35]. MAP0403 or its ortholog is the possible membrane associated serine protease present in pathogenic mycobacteria [35].

**Trypsin-like serine proteases of MAP**

There are four trypsin-like serine proteases in the MAP genome. PepD (MAP0918), PepA (MAP3527) and HtrA (MAP2555c) have been well-studied and characterized. PepD and PepA are culture filtrate proteins. PepD functions as protease and a chaperone [36-38]. MAP0403 is annotated as a hypothetical protein and has not been studied to date.

***M. smegmatis mc$^2$ 155*: A model system**

*M. smegmatis mc$^2$ 155* is a fast growing, non pathogenic mycobateria which is used as a model system to study the biology of virulent and slowly growing mycobacteria [39-44]. Unlike other pathogenic mycobacteria, *M. smegmatis* cannot persist inside the phagosomes [45, 46]. Thus this organism can be used to study the role of virulence genes in macrophage persistence via deletion-complementation approaches.

Classical approaches to fulfill Koch's molecular postulates for gene function use a deletion-complementation approach in the host organism of interest. However, due to constraints in creating targeted deletion mutants in mycobacteria, a model system like *M. smegmatis* is generally applied to study gene function in this group of organisms.

# Chapter 3

## Materials and methods

### Bacterial strains and culture

MAP K10 and MAP 1018 were grown in Middlebrook medium (MB7H9) supplemented with 10% oleic acid-albumin-dextrose (OADC) and mycobactin J at 37°C until the optical density at 600nm reaches 0.3. Cultures were tested for purity using IS*900* PCR and IS*1311* PCR-RFLP analyses. *M. smegmatis mc*$^2$ 155 and the transformants were grown in Luria-Bertani (LB) medium. 100ug/ml hygromycin was added when necessary.

### *In vitro* acid stress

Actively growing (0.3 at OD600) MAP K10 was vortexed for 5 minutes and was passed 10 times through an 18G needle and a sterile syringe to get a homogeneous suspension of the culture without bacterial clumps. The culture was allowed to stand for 5 minutes to facilitate the sedimentation of bacterial debris and dead or clumped bacteria. The upper two-thirds of the culture (containing a single cell suspension) was transferred to a new sterile tube and was used in all subsequent experiments. Acidity of the culture was adjusted to a pH of 5 by 2N HCl. Tubes were inverted thrice to mix the cultures and pH-adjusted cultures and untreated controls were incubated at 37°C for 10, 30 and 120 minutes.

**Macrophage and MAC-T cell staining for confocal microscopy**

Monocyte derived macrophage (MDM) derived from bovine blood as described [11], was seeded at a concentration of 2 x$10^4$cells/well on a 24 well plate containing 1mm thickness glass cover slips and grown in RPMI medium with 10% autologous serum. Cells were incubated for 24 hours at 37°C. Adherence of cells to the cover slip surface was monitored by a phase contrast microscope. Cells treated either with or without bafilomycin at a final concentration of 50 nM(A.G. Scientific Inc) were infected with the GFP-MAP K10, as described [47]. Cells were stained with CellMask (Invitrogen) and Lyso Tracker Blue DND-22 (Invitrogen) according to the manufacturer's specifications and were stored at 4°C until visualized. Olympus Fluoview 1000 confocal microscope (Olympus, South-end-on-sea, Essex) was used to visualize infected and control cells.

**Macrophage and Mac-T cell infection study**

Monocyte derived macrophage (MDM) infection assay was performed as described [47, 48]. Briefly, 2X$10^6$ MDMs were seeded on a T25 flask and incubated at 37°C with 5% $CO_2$. Non-adherent cells were removed by washing with 1X PBS prior to infection. MAP K10 or MAP strain 1018 was spun at 500g for 15 min and re-suspended in RPMI containing 2% autologous serum. The infection medium was mixed well and passed through an 18G needle and syringe to break the clumps. MDMs were infected with MAP K10 or strain 1018 at an MOI of 1 macrophage: 10 bacteria and incubated at 37°C for 2hours. At the end of 2 hours, unphagocytosed bacteria were washed out thrice with pre-warmed PBS and incubated at 37°C with fresh medium for 10, 30 and 120 minutes. As described above, another set of MDMs was infected with *M. smegmatis* $mc^2$ 155, *M. smegmatis* $mc^2$ 155 with the vector pSM417 alone or *M. smegmatis* $mc^2$ 155 carrying

MAP0403 open reading frame. At the end of 2 hours, the cells were washed with PBS and treated with amikacin to kill the extracellular bacteria and incubated at 37°C for respective time points. Macrophages were lysed using 0.1% Triton X100. Lysate was serially diluted and plated on LB agar containing hygromycin (100ug/ml) to determine colony forming units (CFUs).

**RNA extraction**

RNA from the bacterial pellets were extracted using 1ml of TRIzol (Invitrogen, Carlsbad, CA). Bacterial pellet suspended in 1ml of TRIzol was mixed with 0.5ml of sterile RNase free 0.1mm zirconium beads and beat beaded by MagNALyser (Roche). RNA was extracted according to manufacturer's protocol with minor modifications. In brief, the sample was incubated at -20C for 8 hours after mixing with chloroform. Macrophages infected with MAP were lysed using 1 ml TRIzol. RNA was extracted using the previously mentioned method. RNA samples were treated with Turbo DNA Free (Ambion) and subjected to a conventional PCR to confirm that the samples are devoid of genomic DNA. Quality of the RNA was checked by measuring 260/280 ratio on a NanoDrop ND 1000.

**Real Time RT PCR**

One-step real time RT PCR was performed using QuantiFast SYBR Green mix (Qiagen) in a LightCycler 480 (Roche). Untreated broth cultures were used as a base line and *secA* was used as a housekeeping gene. Expression levels were calculated by $2^{-\Delta\Delta CT}$ method [49].

**Microarray Hybridization**

RNA extracted from the macrophage infection were processed and hybridized as described [33]. Briefly, the total RNA extracted and treated with DNase was processed to remove host RNA and the 16s ribosomal RNA. Microbial RNA was amplified using MessageAmp™ (Ambion). Labeled (Cy3 or Cy5) DNA was produced from microbial RNA via first strand cDNA synthesis with aminoallyl-dUTP followed by a coupling of the aminoallyl groups to either Cyanine-3 or Cyanine-5 (Cy-3/Cy-5) fluorescent molecules. In order to get high concentration of labeled DNA, several of the same cDNA reactions were pooled. Effective labeling was achieved by incubating the aminoallyl dUTP coupled cDNA with the dye for 2hours at room temperature. Labeled cDNA was used for hybridization.

**Microarray data analysis**

Hybridized microarray slides were scanned by HP Scanarray 5000 (Perkin Elmer Inc., Waltham,MA). Preliminary image analysis followed by normalization by global lowess was performed by microarray image analysis software BlueFuse (BlueGnome Ltd, Cambridge). Normalized ratios were reported as fold change.

**Construction of *M. smegmatis mc$^2$ 155* with membrane MAP serine protease**

The ORF of MAP0403 was PCR amplified from MAP-K10 genomic DNA using the primers 0403-HF (5' CCC AAG CTT GTG ACG CAC TCG AAT GA 3'), 0403-SR (5' ACA TGC ATG CTC AAC TGA CGC AGG A 3') carrying Hind III and Sph restriction sites at the 5' end. The amplified fragment was cloned into a pSM417 vector (kindly provide by Marcela Rodriguez and Issar Smith, Public Health Research Institute, New Jersey) at

the HindIII and Sph site. Insert orientation and accuracy were confirmed by nucleotide sequencing. pSM417-0403 was electroporated using a single pulse generated at 2500v, 200Ω into competent *M. smegmatis mc*$^2$ 155.

**Fluorescent labeling of *M. smegmatis* transformants**

Actively replicating *M. smegmatis mc*$^2$ *155* and the transformants were stained with 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester (Sigma, 21878) as described Gaggia et al [50, 51]. Briefly, Cells (5ml) were harvested by centrifugation at 13,000 rpm for 5 min and re-suspended in 980µl sterile-filtered citric acid–phosphate-buffer (pH 7.0) to which 10-µl of 1 M glucose and 10-µl of 4.46-mM Carboxyfluorescein N-hydroxysuccinimide ester were added and the solution was incubated at 37 °C for 60 min. Stained cells were harvested by centrifugation at 13,000 rpm for 5 min and resuspended in LB medium (pH~7).

**Establishment of standard curve for fluorescence as a function of pH**

Carboxyfluorescein stained *M. smegmatis mc*$^2$ *155* and the transformants were incubated with ethanol (63% v/v) at 37°C for 30 min to irreversibly permeabilize the membrane. Subsequently, the cells were harvested by centrifugation at 13,000 rpm for 5 min. Cells were resuspended in pH adjusted LB medium (5-ml) ranging from pH 4 to 8 and incubated at 37°C for 30 min. to equilibrate intra-cytoplasmic pH to media pH. Fluorescence at each pH was measured using SpectraMax 2000 (Molecular Probes, San Diego, CA) and the average fluorescence units were plotted against pH.

**Determination of fluorescence of *M. smegmatis* transformants in response to pH variation in vitro**

Carboxyfluorescein stained *M. smegmatis* transformants were resuspended in pH adjusted LB medium (5ml) ranging from pH 4 to 8 and incubated at 37 °C for 30 min. Fluorescence at each pH was measured by Spectramax 2000 at 492 nm excitation and 517 nm emission. The average fluorescence units were plotted against pH.

**Statistical Analysis**

The results pertaining to relative fold changes and CFUs were analyzed by two-way analysis of variance with Bonferroni correction. P-values of less than 0.05 were considered to be statistically significant. Fitness of *M. smegmatis* transformants is shown as box-whisker plots to demonstrate the actual distribution of the observed CFUs.

# Chapter 4

# Results

## MAP0403: A membrane serine protease

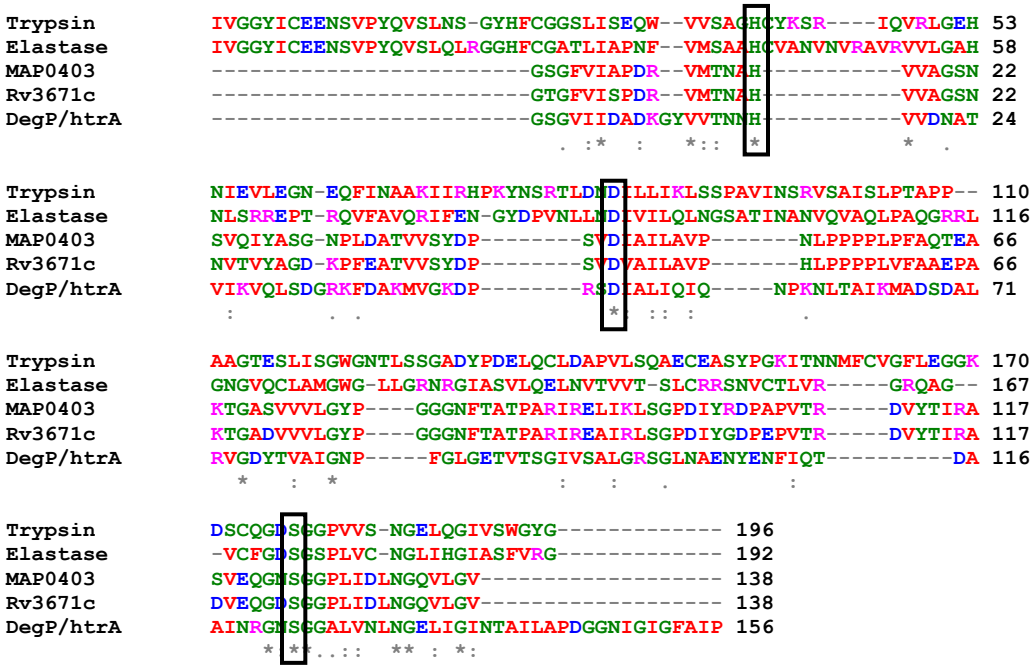
MAP0403 is a 40.6K Da protein composed of 397 amino acids. Comparison of the amino acid sequence of MAP0403 against existing protein databases such as InterPro, Pfam, Prosite and MEROPS revealed that MAP0403 as a homolog of trypsin-like serine proteases. The protease domain is on the C-terminal and the conserved catalytic residues of serine protease namely, histidine, aspartate and serine are located at positions 240, 269 and 348 respectively. Analysis by PSORT, TMHMM and SOSUI identified an N- terminal transmembrane domain composed of 4 helices. MAP0403 is annotated as a hypothetical protein. Based on the amino acid sequence similarity and identification of conserved protease domain, MAP0403 is referred to as ‘serine protease’ in this thesis.

**Figure 1: Topology and motifs of MAP0403:** MAP0403 is composed of 397 amino acids. The transmembrane domain on the N-terminal is composed of four helices. A serine protease domain is located on the C-terminal.


Trans membrane domain

Protease domain

**Figure 2: MAP0403 contains conserved catalytic triad of serine protease.** Amino acid sequence alignment of serine protease domain of MAP0403 with Rv3671c and other well-characterized eukaryotic and prokaryotic serine proteases: human trypsin-2, human neutrophil elastase and *Escherichia coli* HtrA. Boxes indicate the conserved active site residues.


Trypsin IVGGYICEENSVPYQVSLNS-GYHFCGGSLISEQW--VVSAGHCYKSR----IQVRLGEH 53
Elastase IVGGYICEENSVPYQVSLQLRGGHFCGATLIAPNF--VMSAAHCVANVNVRAVRVVLGAH 58
MAP0403 ----------GSGFVIAPDR--VMTNAH----------VVAGSN 22
Rv3671c ----------GTGFVISPDR--VMTNAH----------VVAGSN 22
DegP/htrA ----------GSGVIIDADKGYVVTNNH----------VVDNAT 24
Trypsin NIEVLEGN-EQFINAAKIIRHPKYNSRTLDNDILLIKLSSPAVINSRVSAISLPTAPP-- 110
Elastase NLSRREPT-RQVFAVQRIFEN-GYDPVNLLNDIVILQLNGSATINANVQVAQLPAQGRRL 116
MAP0403 SVQIYASG-NPLDATVVSYDP--------SVDIAILAVP-------NLPPPPLPFAQTEA 66
Rv3671c NVTVYAGD-KPFEATVVSYDP--------SVDVAILAVP-------HLPPPPLVFAAEPA 66
DegP/htrA VIKVQLSDGRKFDAKMVGKDP--------RSDIALIQIQ-----NPKNLTAIKMADSDAL 71
Trypsin AAGTESLISGWGNTLSSGADYPDELQCLDAPVLSQAECEASYPGKITNNMFCVGFLEGGK 170
Elastase GNGVQCLAMGWG-LLGRNRGIASVLQELNVTVVT-SLCRRSNVCTLVR-----GRQAG-- 167
MAP0403 KTGASVVVLGYP----GGGNFTATPARIRELIKLSGPDIYRDPAPVTR-----DVYTIRA 117
Rv3671c KTGADVVVLGYP----GGGNFTATPARIREAIRLSGPDIYGDPEPVTR-----DVYTIRA 117
DegP/htrA RVGDYTVAIGNP-----FGLGETVTSGIVSALGRSGLNAENYENFIQT----------DA 116
Trypsin DSCQGDSGGPVVS-NGELQGIVSWGYG---------- 196
Elastase -VCFGDSGSPLVC-NGLIHGIASFVRG---------- 192
MAP0403 SVEQGDSGGPLIDLNGQVLGV---------- 138
Rv3671c DVEQGDSGGPLIDLNGQVLGV---------- 138
DegP/htrA AINRGDSGGALVNLNGELIGINTAILAPDGGNIGIGFAIP 156

**Gene expression of MAP0403, a serine protease of *Mycobacterium avium* subsp. *paratuberculosis* corresponds with low pH**

MAP parasitizes macrophages and persists in a hostile phagolysosome in an acidic (pH~5) environment. We asked whether MAP serine protease is up regulated during in-vitro acid stress by exposing MAP-K10 to lower pH in culture media. Compared to untreated control (~pH6.8), MAP-K10 exposed to an acidic media (~pH5) for 10 minutes, produced threefold increased amount of serine protease (MAP0403). Although, the test tube simulated acid stress was not the same acid stress encountered by the MAP in the phagosome, these results establish that MAP0403 transcripts were up regulated within 10 min. of exposure to low pH.

**Increased expression levels of MAP0403 correlates with the timing of MAP containing phagolysosomal acidification**

Confocal microscopy was used to monitor the modulation acidification of MAP containing phagosomes over time and to confirm the effect of bafilomycin in blocking phagosomal acidification. MDMs infected with GFP MAP-K10 were stained with cell Mask and Lysotracker Blue. CellMask was used to label the cytoplasmic membrane of MDMs and Lysotracker Blue, an acid sensitive stain was used to label acidified phagolysosome. Findings show that the acidification of the MAP-K10 containing phagolysosomes was completely blocked by bafilomycin treatment. While the MAP-K10 containing phagolysosome of control MDMs acidified in 10 min p.i and highest level of acidification was observed at 30 min p.i. MAP0403 was up regulated in the acidified phagolysosomes at 10, 30 and 120 min. There was a gradual reduction in the

expression levels of MAP0403 from 10 to 120 min. Inhibition of phagolysosomal acidification abrogated the expression of MAP0403. Strain 1018, which is also a clinical isolate, produced increased amounts of MAP0403 transcripts within acidified phagolysosomes compared to the non acidified phagolysosomes.

MAP crosses the intestinal epithelial barrier before it is engulfed by the intestinal macrophages. MAP has to traverse through an acidified phagolysosome within the epithelial cells as well. We used a bovine mammary epithelial cell line, Mac-T cells, to study the expression pattern of MAP0403 in response to acidification. Mac-T cells were infected with GFP MAP-K10 in the presence or absence of bafilomycin. Acidification of infected phagosomes was rapid and peaked in 30 min p.i. and decayed by 120 min p.i. Acidification of phagolysosome was inhibited by bafilomycin treatment. These data establish that up regulation of MAP0403 correlates with phagosomal acidification in the epithelial cells.

**Modulation of gene expression by acid stress**

MAP serine protease is not the only gene differentially expressed during phagosomal acidification [32, 33, 48]. Whole-genome microarray analysis was used to identify genes that are differentially expressed during phagosomal acidification. Protein- protein interaction models were derived using STRING 8, to identify proteins associated in pathways central to MAP0403 expression. Four genes located immediately up stream of MAP0403 (MAP0399c, MAP0400, MAP0401 and MAP0402), mutY, rlmN, MAP2439c and MAP3922 were predicted to be co expressed with MAP0403. It is likely that these

upstream genes are under the regulation of the same promoter or regulator. More directed studies will need to be performed to confirm if this gene expression is polycistronic. Ribosomal large subunit methyltransferase (rlmN) is important for protein synthesis. Acid stress damages the DNA [27]. mutY is involved in DNA repair by base pair excision mechanisms. Gene ontology revealed that MAP0399c and MAP0401 are involved in an interaction network critical to the synthesis of cofactors. Cofactors are important for most of the enzymatic activity. The interaction network shows that the bacterium is actively synthesizing many proteins to combat acid stress.

Microarray results show that a set of genes that are predicted to be associated with serine protease is expressed in acidified phagosomes and the results were confirmed by RT-PCR.

**Figure 3: Up-regulation of MAP0403 during in vitro acid stress.** Fresh inocula of actively replicating MAP-K10 culture in 7H10 broth was exposed to an acidic medium and transcription was analyzed by qRT-PCR for the differential expression levels of MAP0403. Compared to control (pH~6.8), MAP0403 was up regulated during in-vitro acid stress (pH~5) by MAP K10.

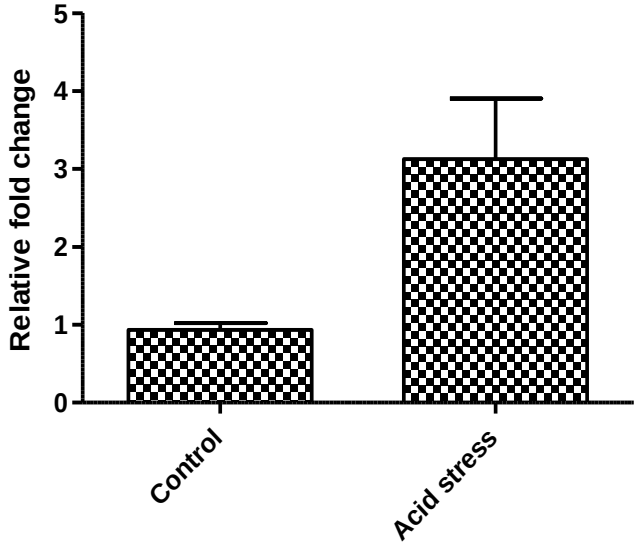

Relative fold change
5
4
3
2
1
0
Control
Acid stress

**Figure 4: Temporality of phagosomal acidification in monocyte derived macrophages (MDMs) infected with green fluorescence protein (GFP) expressing MAP K-10 strain.**

MDMs treated either with 50 nM bafilomycin or vehicle control were infected with GFP expressing MAP-K10 to monitor the time dependant phagosomal acidification of MAP containing phagosome. Cell membrane of MDMs was stained with CellMask (Red). MAP-K10 is visualized in green. Phagosomal acidification was detected by a pH sensitive fluorescence stain - Lysotracker Blue (blue). MAP containing phagosome of the controls acidified at 10min p.i. and peaked at 30min p.i. Clearly, acidification was completely abrogated by bafilomycin that blocks the action of V-ATPases at 10, 30 and 120 min.

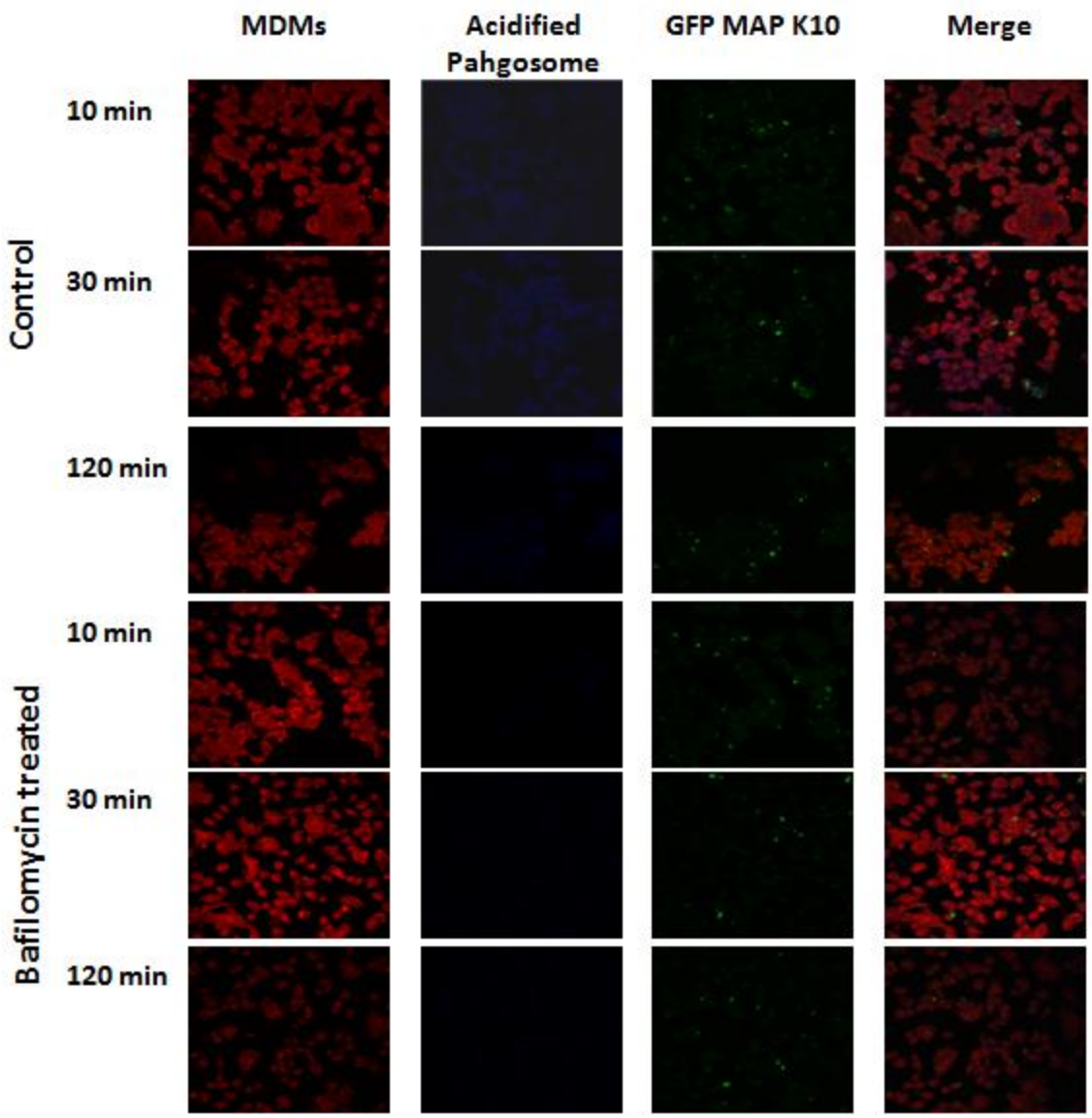

MDMs
Acidified Pahgosome
GFP MAP K10
Merge
Control
10 min
30 min
120 min
Bafilomycin treated
10 min
30 min
120 min

**Figure 5: MAP0403 is up regulated by MAP K-10 in the acidified phagosomes.** MDMs were treated either with 50 nM of bafilomycin or DMSO vehicle control and infected with MAP-K10. RNA was extracted at 10, 30, and 120 min p.i. and examined for MAP0403 expression by qRT-PCR. MAP0403 was up regulated at 10, 30 and 120 min p.i.. Highest level of MAP0403 was observed at 10 min p.i., which gradually decreased with time. Bafilomycin treatment that blocked acidification, significantly ($p<0.0001$) reduced the expression of MAP0403.

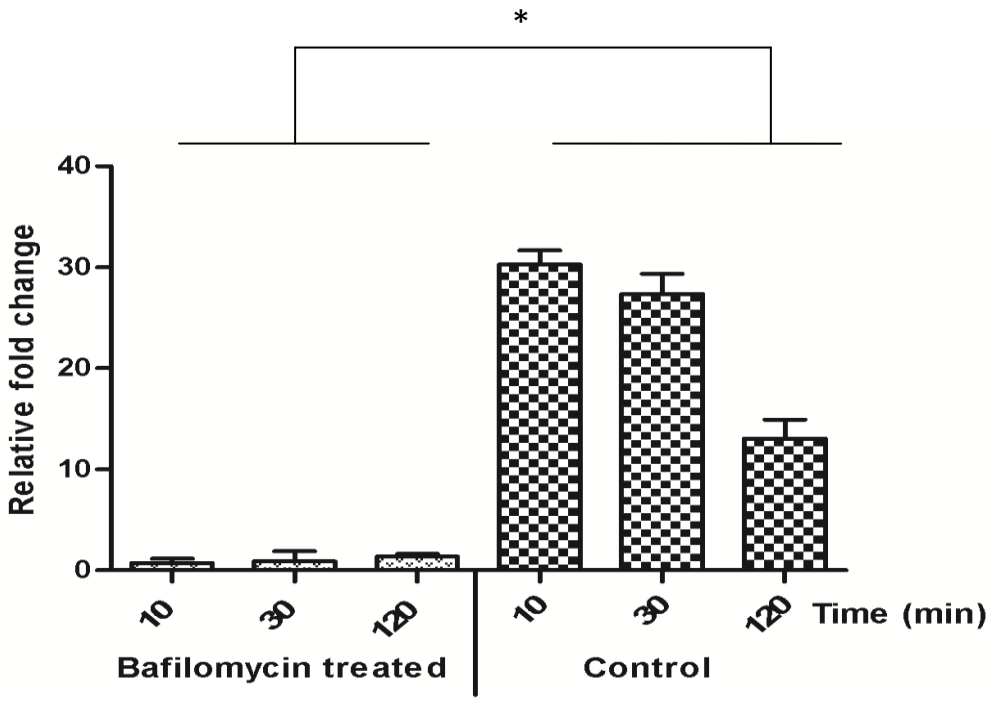

*
40
30
20
10
0
Relative fold change
10
30
120
10
30
120
Time (min)
Bafilomycin treated
Control

**Figure 6: MAP0403 is up regulated by MAP 1018 in the acidified phagosomes.** MDMs were treated either with 50nM of bafilomycin or DMSO vehicle control and infected with MAP 1018. RNA was extracted at 10, 30, and 120 min p.i. and MAP0403 expression was analyzed by qRT-PCR. MAP0403 was up-regulated at 10, 30 and 120 min p.i. Highest levels of MAP0403 was observed at 30 min p.i. Bafilomycin treatment, significantly ($p<0.0001$) reduced the expression of MAP0403.

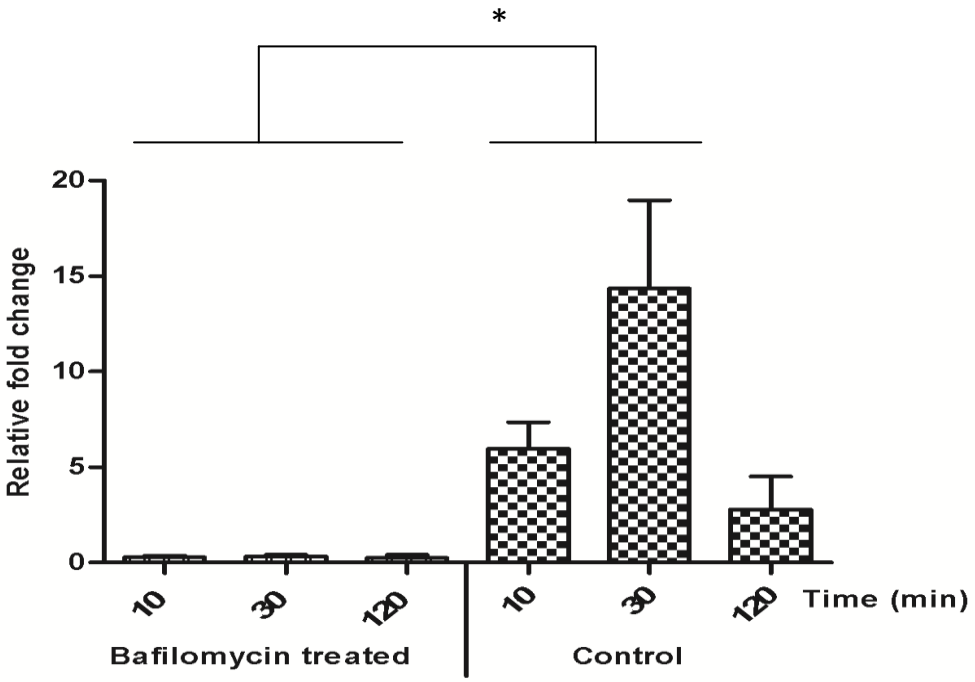

*
Relative fold change
20
15
10
5
0
10
30
120
10
30
120
Time (min)
Bafilomycin treated
Control

**Figure 7: Confocal images of MAP-K10 infected Mac-T cells.** Mac-T cells treated either with 50nM bafilomycin or vehicle control was infected with green fluorescence protein expressing MAP-K10 to monitor the time dependant phagosomal acidification of MAP containing phagosome. Cell membrane of MDMs was stained with CellMask (Red). MAP-K10 was shown in green and acidification of MAP containing phagolysosome was detected by a pH sensitive fluorescence stain, LysotrackerBlue (blue). MAP containing phagosome of the controls mildly acidified at 10min p.i. and intense acidification was observed at 30min p.i. But bafilomycin treatment blocked the acidification of MAP containing phagosome.

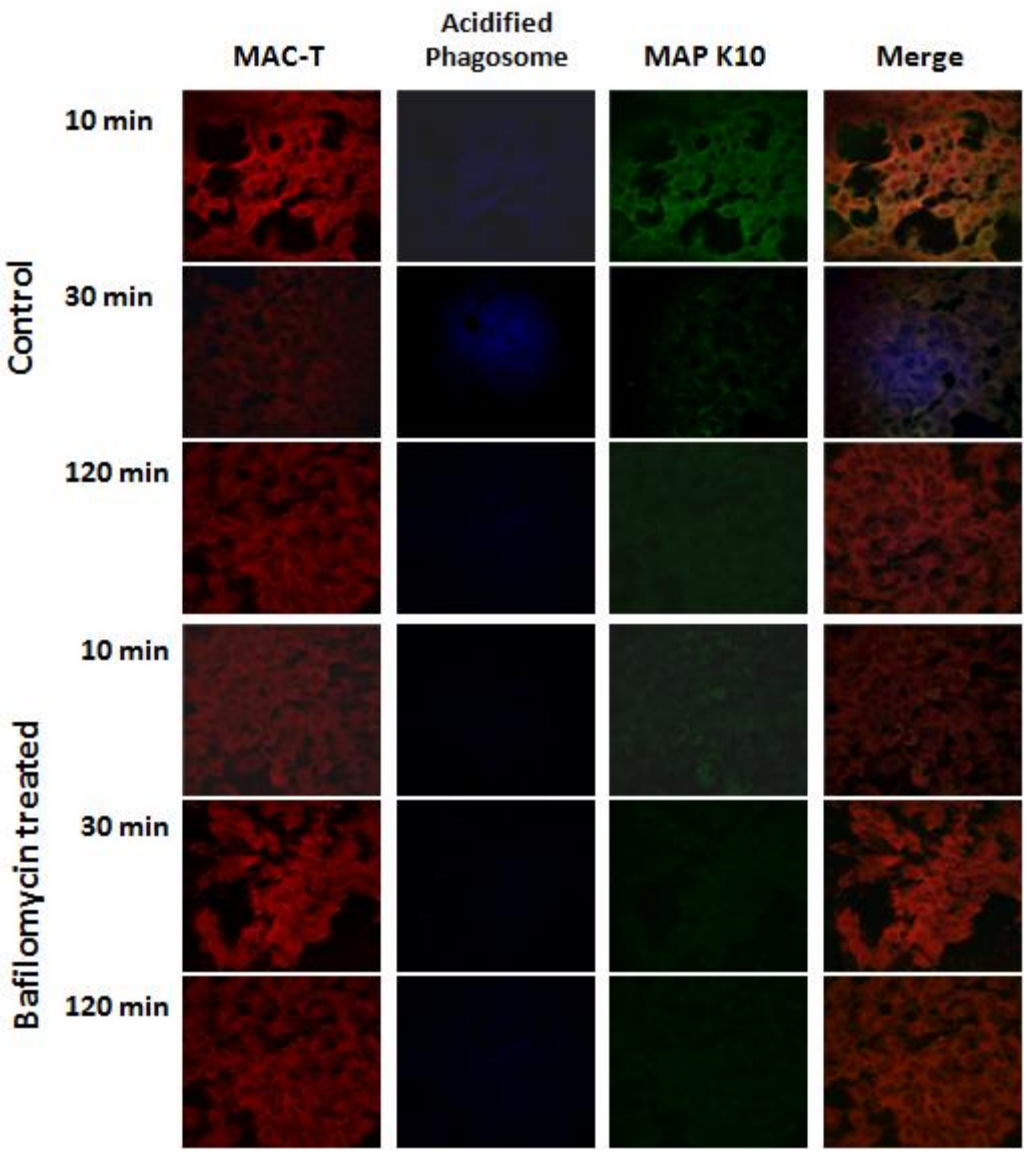

MAC-T
Acidified Phagosome
MAP K10
Merge
Control
10 min
30 min
120 min
Bafilomycin treated
10 min
30 min
120 min

**Figure 8: MAP0403 is up regulated in the acidified phagosomes of epithelial cells.** Mac-T cells were treated either with bafilomycin or DMSO vehicle control and infected with MAP-K10. RNA was extracted at 10, 30, and 120 min p.i. and examined for the increased or decreased levels of MAP0403 expression by qRT-PCR. MAP0403 was up-regulated at 30 and 120 min p.i. Bafilomycin treatment that blocked the acidification significantly ($p<0.05$) reduced the expression of MAP0403.

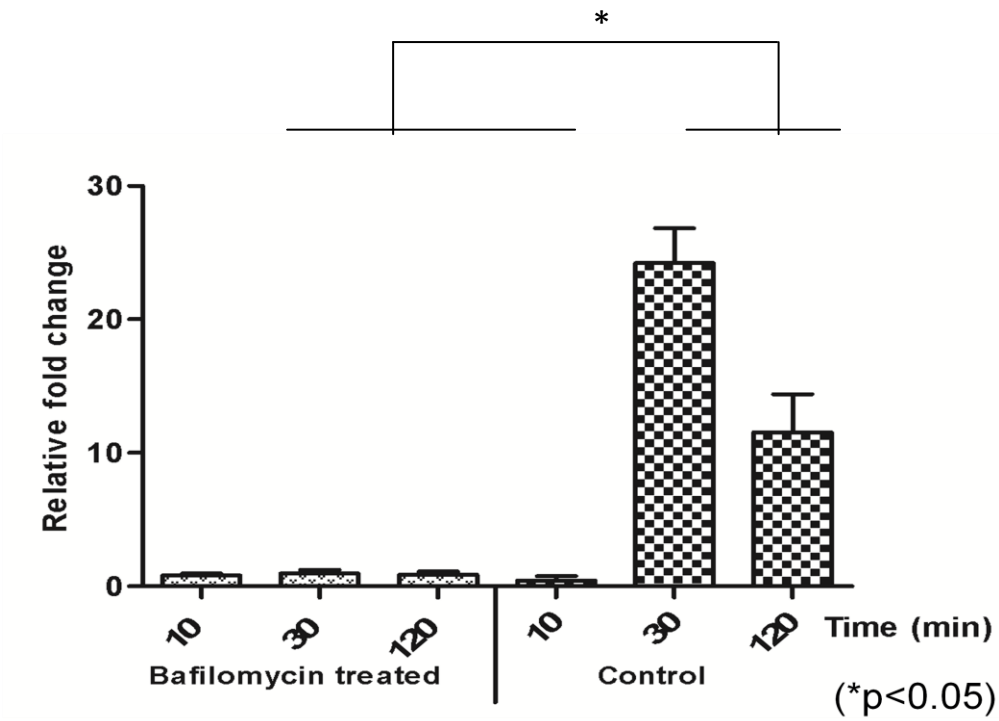

*
30
20
10
0
Relative fold change
10
30
120
10
30
120
Time (min)
Bafilomycin treated
Control
(*p<0.05)

**Figure 9: Predicted protein-protein interaction of MAP0403.** Prediction of protein-protein interactions are made based on experimental repositories, computational prediction methods and public text collections deposited in the STRING database. (http://string-db.org.)

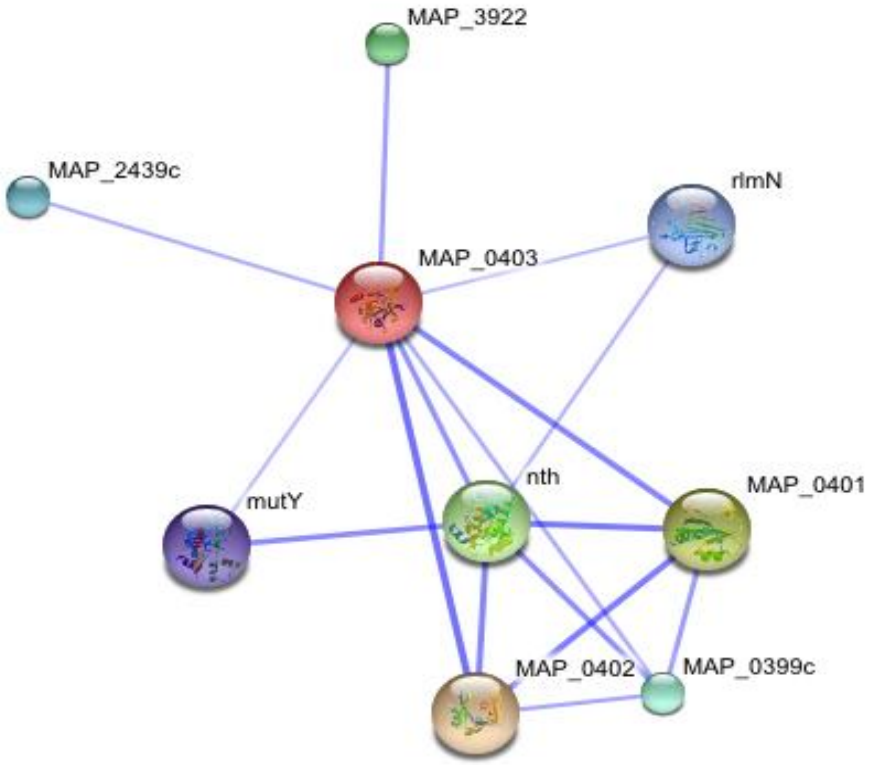
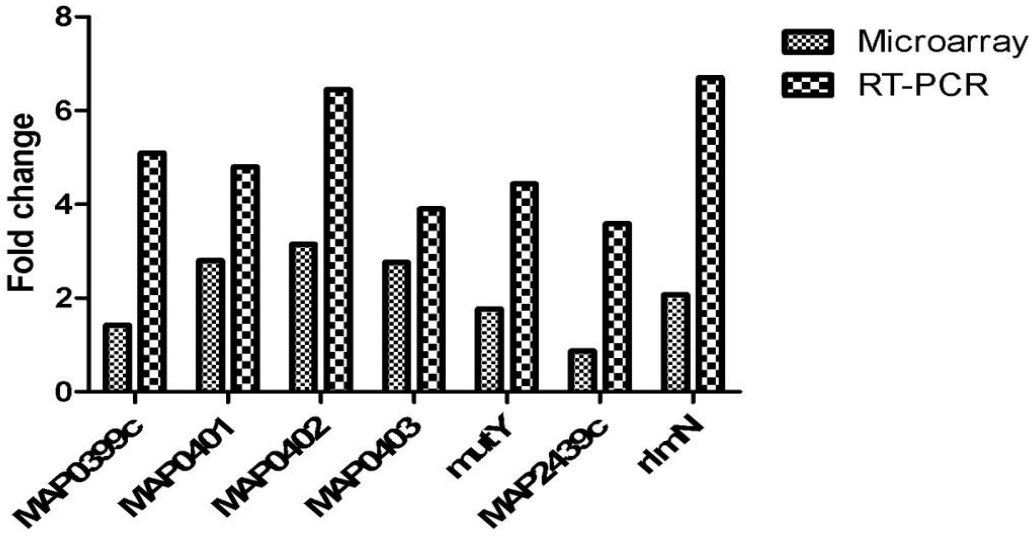

MAP_3922
MAP_2439c
rlmN
MAP_0403
nth
MAP_0401
mutY
MAP_0402
MAP_0399c

**Figure 10: Predicted proteins related to MAP0403 were up regulated during phagosomal acidification.** Proteins predicted to interact with or co-expressed with MAP0403 during phagosomal acidification were screened by microarray. Proteins predicted by http://string-db.org were up regulated in the acidified phagosomes. Microarray results were validated by real-time PCR to demonstrate the similar trends.


Fold change
8
6
4
2
0
Microarray
RT-PCR
MAP0399c
MAP0401
MAP0402
MAP0403
mutY
MAP2439c
rlmN

**MAP0403 ortholog in *M. smegmatis,* MSMEG6183, is not expressed under *in vitro* acid stress**

Unlike pathogenic mycobacteria, non-pathogenic mycobacteria such as *M. smegmatis mc$^2$ 155* cannot survive and persist in the phagolysomomes. Furthermore, *M. smegmatis mc$^2$ 155* cannot resist the phagosomal acidic pH. Therefore, it is important to know how the expression of MSMEG6183, the ortholog of MAP0403, is modulated in response to an acidic environment. The in-vitro acid stress experiment results show that MSMEG6183 was not differentially expressed.

**MAP0403 transformants of *M. smegmatis* survive *in vitro* acid stress**

*M. smegmatis mc$^2$ 155* transformants carrying the MAP serine protease and vector controls were tested for survivability in acidic media (pH~5). Although the acid stress experienced by intra-cellular *M. smegmatis* was not similar to in-vitro acid stress, MAP serine protease complemented transformants survived acid stress relatively better than the controls at 30 and 120 min.

**MAP0403 transformants of *M. smegmatis* resist phagosome acidification in monocyte derived macrophages**

*M. smegmatis mc$^2$ 155* transformants containing the MAP serine protease and vector controls were tested for survivability inside the MDMs. Transformants carrying serine protease showed an increased survival in the phagolysosomes. Compared to time zero,

a 1.5 log and 3 log reductions of bacteria was observed in MAP0403 carrying transformants and controls, respectively.

**MAP0403 transformants maintain intra-bacterial pH in the acidic environment**

Maintaining intra cytoplasmic pH within a physiological range is important for all the cellular process. Compared to the controls, transformants that contain MAP serine protease maintained its intra cytoplasmic pH within the physiological range while exposed to an acidic (pH~5) media.

**Figure 11: MAP0403 helps to resist in-vitro acid stress.** *M. smegmatis* transformants were exposed to an acidic media (pH~5) for 10, 30 and 120 min. Viable bacterial counts were determined by serial dilution and plating on LB agar containing hygromycin (100ug/ml). Viable counts of both transformants reduced at 10 min. but, compared to vector controls, transformants carrying MAP0403 multiplied and survived the acid stress at 30 and 120min.

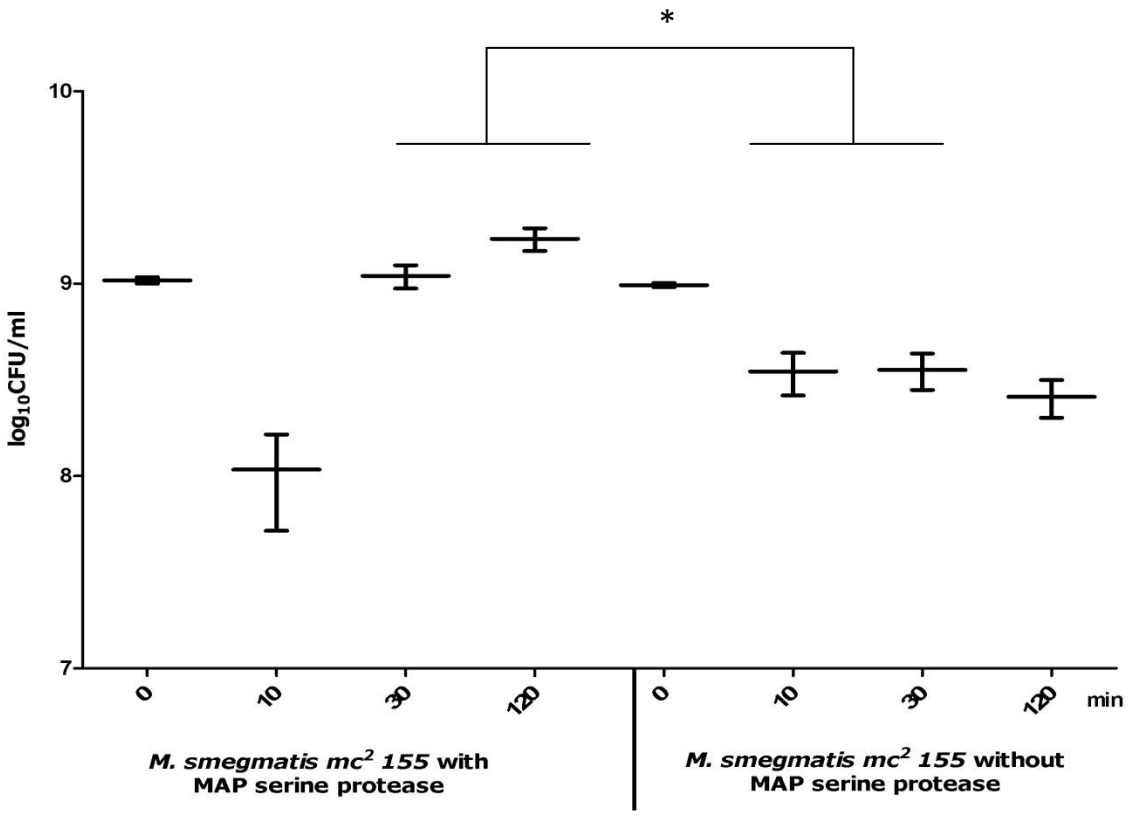

*
10
9
8
7
log10CFU/ml
0
10
30
120
0
10
30
120
min
M. smegmatis mc2 155 with MAP serine protease
M. smegmatis mc2 155 without MAP serine protease

**Figure 12: MAP0403 increases intra cellular survivability of *M. smegmatis* transformants.** MDMs were infected with *M. smegmatis* transformants and viable bacterial counts were determined at 10, 30 and 120 min by serial dilution and plating. MAP serine protease carrying transformants survived significantly greater ($p<0.0092$) than parent strain or vector controls at 10, 30 and 120 min p.i.

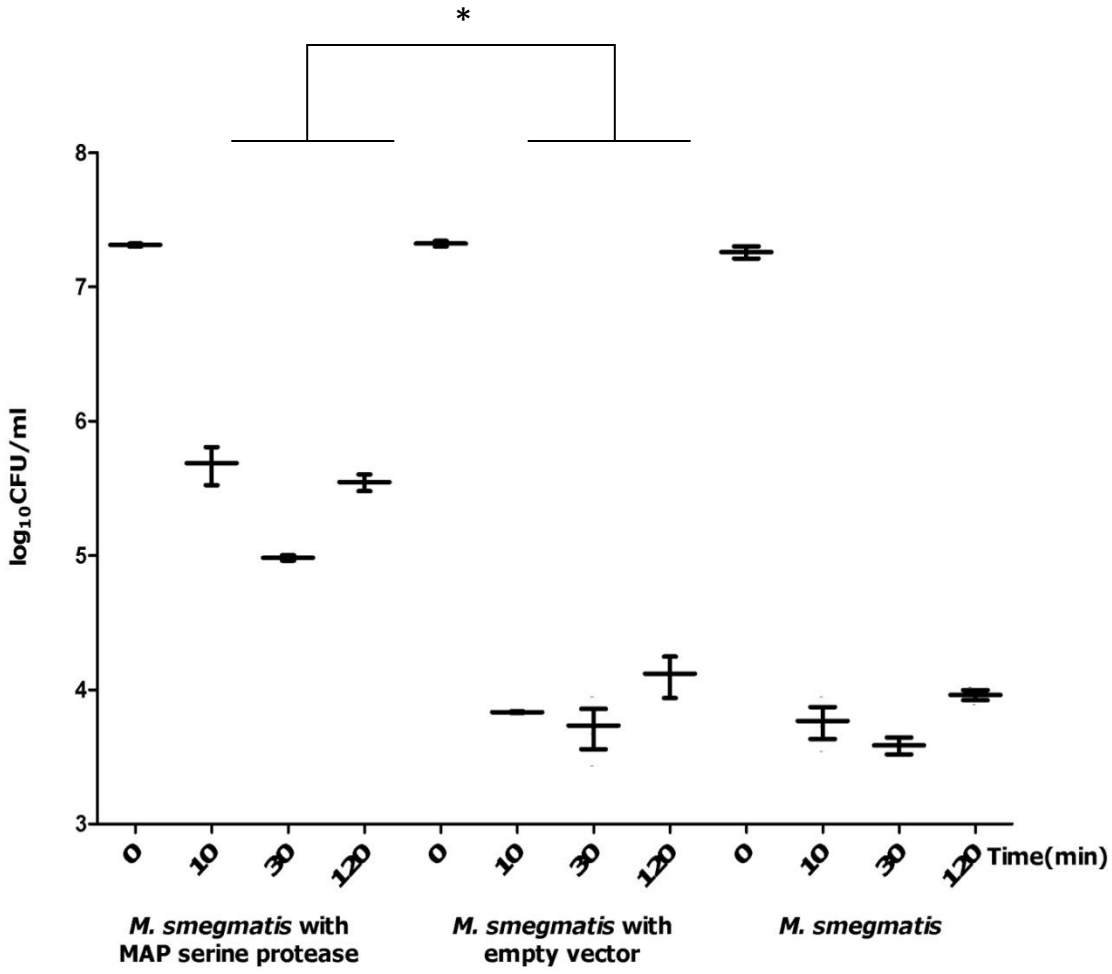

*
8
7
6
5
4
3
log10CFU/ml
0
10
30
120
0
10
30
120
0
10
30
120
Time(min)
M. smegmatis with MAP serine protease
M. smegmatis with empty vector
M. smegmatis

**Figure 13: Calibration curve for measuring intra-cytoplasmic pH of *M. smegmatis* transformants.** *M. smegmatis* transformants were stained with carboxyfluorescein followed by membrane permeabilization and exposure to a range of pH. Fluorescence was measured and plotted against pH. Both transformants stained similarly and did not differ in fluorescence when the intra-cytoplasmic pH was equilibrated to media pH. Fluorescence is proportional to the increasing pH range between pH 5 to pH 8. Relative fluorescence unit was used within the range of pH5 to 7 to indirectly measure the intra-cytoplasmic pH.

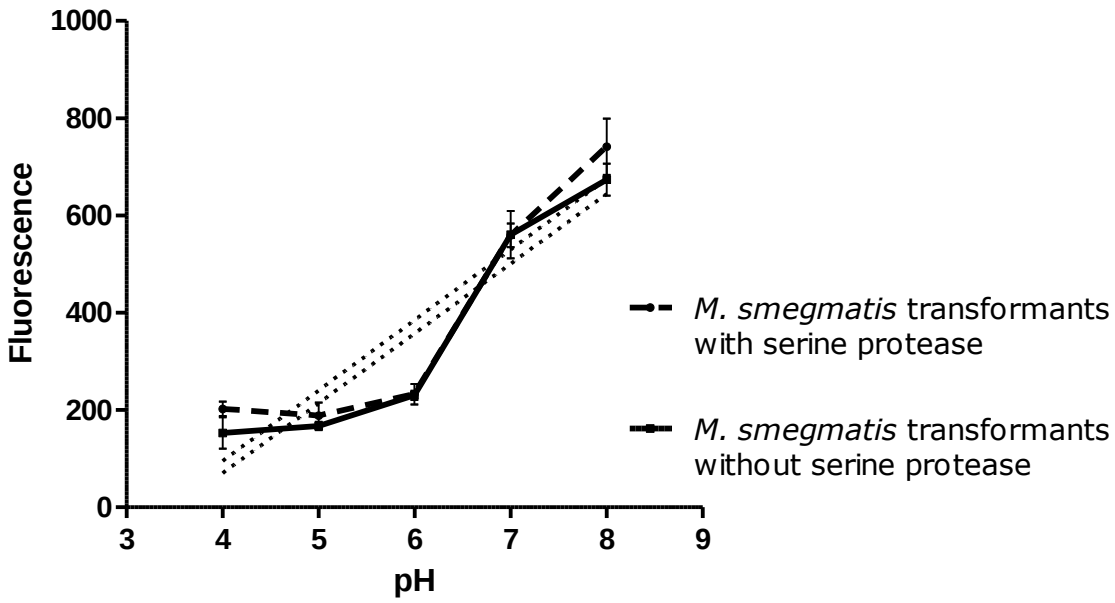
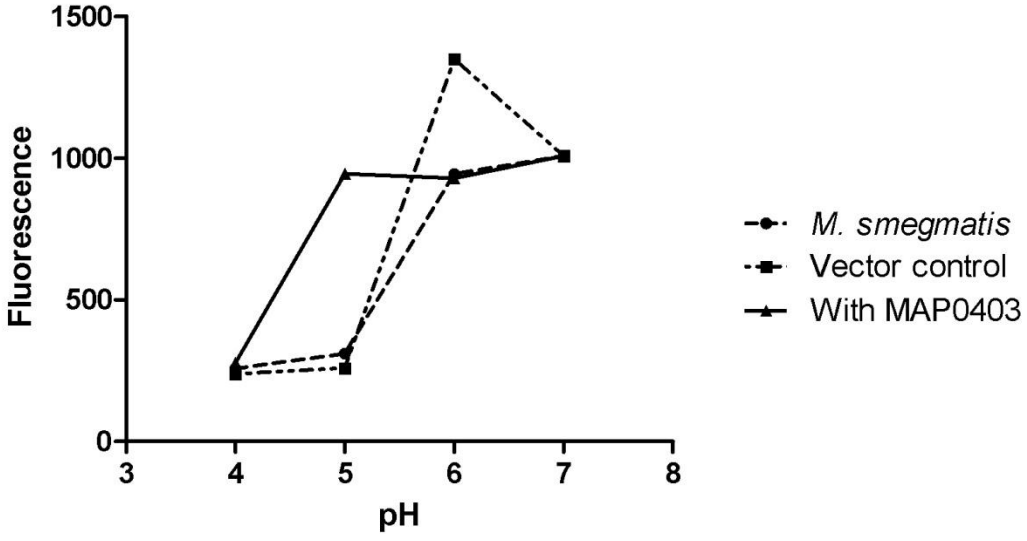

1000
800
600
400
200
0
Fluorescence
3
4
5
6
7
8
9
pH
M. smegmatis transformants with serine protease
M. smegmatis transformants without serine protease

**Figure 14: Differences in intra-cytoplasmic pH homeostasis of *M. smegmatis* transformants.** Intra-cytoplasmic pH of *M. smegmatis* transformants exposed to different acidic media was determined by Carboxyfluorescein staining. Compared to controls, MAP0403 carrying *M. smegmatis* transformants were able to maintain its intra-cytoplasmic pH when exposed to an acidic environment (pH~5).


1500
1000
500
0
Fluorescence
3
4
5
6
7
8
pH
M. smegmatis
Vector control
With MAP0403

## Discussion

MAP containing phagolysosomal compartment is acidic, rich in lysosomal hydrolases, and reactive oxygen and nitrogen intermediates. Understanding the role of specific proteins or groups of molecules that contribute to MAP survival and persistence in this hostile compartment will help improve our understanding of the mechanisms by which MAP persist in the macrophages and aid the discovery of new drug targets for treatment and prevention. Of the four, trypsin like serine proteases present in the MAP K-10 genome, studies have addressed the importance of *htrA*, *PepA*, and *PepD,* while the role of MAP0403 remains elusive [36, 37, 52]. We were interested on MAP0403, an ortholog of Rv3671c that has been recently shown to be critical in maintenance of intra-bacterial pH [1]. Amino acid similarity between MAP serine protease and the recently characterized MTB protease implies that they may also share function under specific physiological conditions.

PDZ (Post synaptic density protein, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein) domain is a structural domain composed of 80 – 100 amino acids [53]. PDZ domains are associated with other protein domains and the combination allows them to carry out specific functions. HtrA, a well characterized trypsin like serine protease in *Salmonella enterica* serovar Typhimurium and *E. coli* has a C-terminal PDZ domain that enhances the protease activity through increased protein-protein interactions [54, 55]. However, both MTB and MAP serine proteases lack the PDZ domain. Thus the kinetics of mycobacterial serine proteases may differ from those that of *Salmonella* and *E. coli*.

Using confocal microscopy we demonstrated that the MAP containing phagosomal acidification occurs as early as in 10 minutes of phagocytosis is in agreement with MTB

studies [24, 56]. Determining the time dependant pH change of MAP containing phagolysosome is important to choose an appropriate time point for the examination of genes that are transcribed in response to immediate acid stress – thus our initial studies established the timing of phagosomal acidification after phagocytosis that enabled us to accurately time the genetic events deployed by MAP in response to pH.

MAP interacts with the intestinal epithelial cells before it is engulfed by macrophages. Although epithelial cells are not the professional phagocytic cells, acidified phagolysosomes are formed within the epithelial cells and the priming in the epithelial cells is important for MAP to orchestrate its evasion from the epithelial cells (Lamont and Sreevatsan, Unpublished). Here we show that MAP containing phagolysosome of an epithelial cell acidifies in 30 min p.i.. Increased expression levels of MAP0403 are noticed in the MDMs and Mac-T cells tightly correlates with the timing of phagosomal acidification. Stress responsive proteins significantly contribute to the fitness of the microorganism during the infection. Our findings suggest that MAP0403 is indeed a low pH responsive protein. However, Wu et al did not find an increased expression of serine protease in response to acid stress in their microarray based studies. The differences in specific gene expression are likely due to the fact that acid exposure time was different than those used by of Wu et al who studied expression after chronic exposure to acidic stress. Our findings suggest that MAP0403 may be a more proximal event that modulates all downstream events in the maintenance of intra bacterial pH. Our findings were also supported by the early appearance of MAP0403 transcripts in a macrophage and epithelial cell infection models. Consistent with our reasoning, microarray analysis identified a 2.76 fold increase of serine protease expression during phagosomal acidification. In agreement with Wu et al, the microarray results show that large numbers of genes (30% genes) were turned on during phagosomal acid stress and 92% of these

were common across the two studies and warrant a comprehensive comparative analysis.

MAP0403, the membrane serine protease is conserved across *Mycobacterium* species. Ninety four percent amino acid similarity was noticed between MTB serine protease (Rv3671c) and MAP serine protease (MAP0403) while 68% amino acid similarity is observed between MAP0403 and its ortholog (MSMEG6183) in *M. smegmatis*. *M. smegmatis* is acid sensitive. Our finding that MSMEG6183 is not differentially expressed during in-vitro acid stress, suggesting that the function or the regulation of expression of MSMEG6183 is likely different than that of MAP0403. Roxas et al studied the proteins of cell membrane and cell wall that are expressed during acid stress [57]. Their finding that MSMEG6183 was not identified as an acid induced protein validates our contention that *M. smegmatis mc$^2$ 155* can be used as a surrogate to study the function of MAP0403 by complementation.

Deletion or truncation mutations of specific gene of interest are often used to understand their function. Creating targeted mutants in genes of pathogenic mycobacteria is challenging. All attempts to create MAP0403 mutant in Jeff McGarvey's and Jim Posey's laboratories at USDA and CDC, respectively, failed. Thus, *M. smegmatis* was used as a surrogate to study MAP0403. Compared to controls *M. smegmatis* transformants carrying MAP0403 survived well in the acidic environment. We expected to see an increased transcription of MAP0403 by *M. smegmatis* transformants under acid stress. But we were not able to detect the difference between the acid exposed and non exposed MAP0403 transformants MAP0403 is cloned immediately downstream of the Hsp60 promoter on the pSM417 vector. Hsp60 is a constitutively expressing promoter [58]. So, irrespective of the acid exposure, MAP0403 transcripts are made even at time

zero. Acid regulated promoters of mycobacteria are not well studied. Further studies should be carried out to confirm mechanisms of regulation of MAP0403 expression in an *M. smegmatis* model.

Mycobacteria are neutrophiles that maintain a near neutral intra bacterial pH [25, 27, 29, 30]. Exposure of *M. smegmatis* to an acidic media (pH~5) reduces the intra bacterial pH and thereby significantly reduces its growth rate [30]. *M. smegmatis*, a non pathogenic mycobacterium is acid sensitive and not able to persist in the macrophages [45, 59]. Stress responsive serine proteases of intracellular pathogens significantly contribute to their survival fitness in the host [1, 60-63]. Our results on parent strain and the vector control are in agreement with the previous findings and emphasize that the introduction of MAP serine protease into *M. smegmatis* conferred protection against acid stress by aiding the maintenance of pH. However, the mechanism by which MAP serine protease confers acid resistance remains to be unidentified. Further, the growth pattern of serine protease carrying transformants during in vitro acid stress and in MDMs resemble a typical pattern of any intracellular pathogen, reiterate the important role of MAP serine protease.

Bovine Monocyte derived macrophages are good models to study MAP-macrophage interaction [11, 21, 47, 48, 51]. The magnitude of the survival advantage of membrane serine protease carrying transformants is more in MDMs than in-vitro acid stress. Intra-phagosomal environment is not the very same as the artificially acidified media. And it is a likely reason for the bacterial survival differences observed during in-vitro acid stress and in MDMs. Thus, our findings also demonstrate the inefficiency of simulating artificial stress conditions and re-emphasize the usage of macrophage model to study MAP pathogenesis.

## Conclusion

In summary, MAP serine protease is an acid responsive protein and its expression correlates with phagosomal acidification. Database search on protein–protein interactions revealed a set of genes that might associate with MAP serine protease. The microarray results confirmed by real time PCR show that these set of genes are up regulated with serine protease. A proteomics approach can be used to further validate these results. Non pathogenic acid sensitive mycobacteria, *M. smegmatis* were able to resist phagosomal acid stress upon complementation with MAP serine protease. Further, the MAP serine protease containing *M. smegmatis* transformants were able to maintain its intra bacterial pH when exposed to an acidic culture medium (pH~5). All these finding suggest that MAP serine protease is important in resistance to phagosomal acid stress. Further studies should be carried out to confirm how MAP serine protease interacts with other proteins to maintain intra-bacterial pH.

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