AN ABSTRACT OF THE DISSERTATION OF

Ida L. Phillips for the degree of Doctor of Philosophy in Comparative Health Sciences presented on July 29, 2020.

Title: Bacterial and Host Factors Contributing to *Mycobacterium avium* subspecies *paratuberculosis* Persistent Infection

Abstract approved:

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic granulomatous enteritis that plagues domestic and wild ruminants globally. During the silent stages of Johne's disease, infected animals intermittently shed bacteria for years prior to clinical diagnosis during advanced disease stages. This strategy allows MAP to persist and spread in the environment and on farms undetected leading to enormous financial loss. There is a need for better understanding to understand many aspects of bacterial pathogenesis in order to successfully control and prevent MAP infection. The research of this dissertation aims to identify a) virulence factors of MAP that contribute to pathogen escape of host killing mechanisms and b) host cellular factors that allow MAP to persist and survive within professional phagocytes.

We describe *Acanthamoeba castellanii* (amoeba) as a MAP infection model of the environmental protozoan host and as a tool for selecting bacterial genetic factors potentially contributing to MAP virulence in host macrophages. Using MAP transposon gene knockout mutants, we demonstrate altered amoeba metabolism and

that associated metabolic changes, identified through screening of MAP transposon gene knockout mutants, which mirror survival patterns and intracellular MAP burdens during macrophage infection. Transposon mutant infections of amoeba led to -infected amoebas display differential (either excessive or decreased) changes in metabolic activity versus those of the compared to wild-type (WT) MAP infection. The majority of mutants identified in the amoeba screen were also found to be growth deficient in Raw 264.7 macrophages. Sequencing and bioinformatic analysis of these mutants help to understand and further characterize pathogen-associated factors that the pathogen most likely uses as a strategy used to persist and grow within host macrophages.

To further our knowledge of host-pathogen interactions, we used an *in vitro* cell culture passage model previously developed in our lab. MAP was found to develop a more pro-inflammatory phenotype after being passed through passage in bovine epithelial cells and macrophages. Utilizing global proteomic analysis of macrophages to analyze cellular metabolic and regulatory pathways, we found promotion of an intracellular phenotype and adaptation within phagocytic cells during different stages of infection. In this dissertation, we show that direct infection of phagocytes by plate growth MAP (direct infection) leads to a robust pro-inflammatory protein profile, metabolic and cellular targets of anti-inflammatory pathways are being highly synthesized when while MAP is first passaged through epithelial cells and before the infection of prior to infecting macrophages (direct infection) upregulates metabolic and cellular targets of anti-inflammatory pathways. Ultimately, specific protein ontology groups including extracellular matrix proteins

(integrins) and intracellular organelle coordination proteins were identified. An integrin binding assay and the transwell migration assay confirm enrichment of ECM protein production in macrophages that lead to increased binding and transmigration through the endothelium monolayer.

Together, two modeling systems used in this dissertation identified specific host and pathogen factors that significantly expanded our understanding of bacterial genetic factors and phagocytic cell signaling pathways that may allow extended MAP survival within a host. These findings provide the framework to develop strategies for elimination, prevention and diagnostics of Johne's disease.

Bacterial and Host Factors Contributing to *Mycobacterium avium* subspecies *paratuberculosis* Persistent Infection

by
Ida L. Phillips

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Ida L. Phillips, Author

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DEDICATION

I dedicate this dissertation to all the Black men, women, and children who have lost their lives to the beast of white supremacy, capitalism, and patriarchy. You will never be forgotten. Your name lives on. No justice. No peace.

Chapter 1 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of Johne's Disease (JD), a chronic, progressive, granulomatous enteritis afflicting ruminant animals. MAP was first observed by Johne & Frothingham in 1895, but Koch's postulates were not successfully fulfilled by until 1910 by F.W. Trowt (Chiodini et al.). The bacterium is widely disseminated, encompassing Europe, Australia, New Zealand, Japan, India, and the United States (R. Whittington et al.). Global losses associated with this infection range from 200 million to 1 billion dollars per year (Good et al). The dairy industry is most significantly affected with 68-91% of animals having positive serological titers (Otts et al). The economic costs and its negative effect on animal health are significant due to premature culling and reduced milk and meat production due to reduced body condition (Cho et al.). Lombard at el. found that management changes are designed to reduce MAP infections in addition to the culling of clinically affected animals in order to reduce the prevalence of MAP at a herd-level (Lombard et al.). Infertility and early fetal loss are also associated with seroconversion for MAP in high-producing dairy cows (Garcia-Ispierto and López-Gatius). Animals suffering from Johne's disease are predisposed to other conditions for which they are commonly culled. These diseases include ketosis, lameness, mastitis, displacement abomasum, injury, metritis, milk fever, pneumonia, and retained placenta (Raizman et al.), Other costs, are associated with replacement of animals, diagnostic test administration. Diagnostic testing and veterinary care also contributes to the loss of profit for producers (R. Whittington et al.).

MAP is predominantly established in domestic (cattle, sheep, goats, camelids) and wild (deer and antelope) ruminants (Cunha et al.). Non-traditional species found to have lesions associated with Johne's disease include weasels, foxes, horse, and pig (Greig et al., 1999).

MAP is a slow-growing (20 hours or more generation time *in vitro*), acid-fast, Gram-positive, facultative intracellular pathogen (Rathnaiah et al.). *Mycobacterium tuberculosis* and other mycobacterial species have a thick, waxy cell wall. This "sturdy hydrophobic barrier" makes them difficult to control (Thanna and Sucheck). The cell wall is composed of a plasma membrane, a peptidoglycan layer, glycans with mycolylarabinogalactan glycolipids, and an outer capsule (Catalão et al.). Trehalose is an essential metabolite in mycobacteria, playing key roles in cell wall synthesis, transport of cell wall glycolipids, and energy storage (Thanna and Sucheck). Thanna et.al. found that trehalose protects the bacteria from stressors such as desiccation, freezing, starvation and osmotic stress.

The mycobacterial species *M. avium* is currently subdivided into four subspecies, *M. avium* subsp. *avium, M. avium* subsp. *hominuis (MAH), and M. avium* subsp. *silvaticum.* MAP can be differentiated phenotypically from *M. avium* and *M. silvaticum* by its dependence on mycobactin, an iron-binding siderophore isolated from *Mycobacterium phlei* (Thorel et al.) and its presence of multiple copies of an insertion element, IS900 (Thoresen and Olsaker). Whole-genome sequencing of MAP K10 bacterial strain found that the homolog of mbtA ,which encodes for mycobactin, is truncated in MAP (Wang et al.). This could potentially lead to inactivating mycobactin production.

Etiology and Pathogenesis

The pathogenesis of the MAP infections begin when calves six months or younger ingest the bacterium in feces, contaminated milk, feed, or water (Streeter et al.). Studies by Windsor at el. show that ruminant neonates exposed to MAP at the time of birth have an increased risk of infection progressing to Johne's disease,, particularly in highly contaminated environments or if the dam is infected (Windsor and Whittington). However, calves around 6 months of age have a lower risk of infection coinciding with declining numbers of Peyer's patches (Jung et al.). Experiments using Peyer's patch-deficient mice found that MAP uses enterocytes lining the mucosa as well as microfold cells (M cells) to enter the lamina propria (Bermudez et al.). Light and electron microscopic studies established that MAP can invade the cytoplasm of intraepithelial leukocytes and M cells less than one hour after injection into the intestinal lumen (Sigurðardóttir et al.).

Once the bacterium is ingested, the host immune system engages bacterial factors to overcome the host defenses(Arsenault et al.). MAP invasion of the gut lining and survival in macrophages are two critical stages that determine if the animal will clear the infection or if the animal will develop stages of the Johne's Disease (Arsenault et al.). Within each infection stage, MAP encounters a variety of host defense systems that must be averted or subverted for the pathogen to establish infection. Johne's disease is often categorized into 4 stages, including: 1) silent infection, 2) subclinical infection, 3) clinical, and 4) advanced clinical disease. Stages are based on the severity of clinical signs and potential shedding of organisms into the environment. The gross lesions of Johne's disease lesions are most prominent

in the ileum, large intestine, and draining lymph nodes. During advanced clinical stages, the infection spreads to all parenchymatous organs via the lymph and blood circulation. One of the difficulties in controlling MAP infection is asymptomatic animals that frequently shed MAP in feces over time. The average incubation period is unpredictable but estimated at 5 years. This is a contributing factor to infection spread amongst herd mates.

It is not fully understood how MAP successfully navigates the forestomach of ruminant species and overcome intestinal barriers such as IgA secretion, antimicrobial peptides, and mucus layers to end up in the lamina propria macrophages (Janagama et al). It is believed to access mucosal macrophages/dendritic cells by transmigration through the enterocytes overlying the epithelium and through microfold which has been experimentally demonstrated in mice. MAP recognizes and binds to a variety of receptors. Fibronectin binding and $\beta 1$ integrins are thought to be required for attachment and internalization of MAP *in vitro* and *in vivo* (Viale et al.) How long MAP remains in macrophages or how these enterocytes alarm immune cells to the site of infection remains unknown. Once MAP leaves the enterocytes, local phagocytes take up the bacterium. Macrophages are the preferred host cell for MAP, therefore, undermining this professional immune cell's well-coordinated and abundant killing mechanisms is essential for MAP survival, persistence, and spread (Sweeney). Macrophages and dendritic cells must first recognize the invading pathogen to mount an immunogenic attack. Macrophages recruited to a site of infection or injury display a pro-inflammatory phenotype, secreting a variety of inflammatory mediators such as tumor necrosis factor-α, IL-1, IL-12, and IL- 23 (F.

Delgado et al.). This cytokine signaling leads to a robust T-cell response designed to resolve MAP infection. In response, mycobacterial species suppresses macrophage activation to promote bacterial survival and persistence (Sia and Rengarajan). Mechanisms used by mycobacterial species to survive within professional phagocytes are not limited to suppression of antigen presentation (Harding and Boom), antigen processing by MHC expression, induction or prevention of apoptosis for infected macrophages (Kabara and Coussens), absent or ineffective nitric oxide (Zhao et al.) or superoxide dismutase production (Liu et al.), and poor interferon gamma signaling (Khalifeh et al.). These methods are used by mycobacterial species to promote survival within professional phagocytes. Other strategies employed by mycobacteria to avoid enzymatic and toxic attack within macrophages include avoidance of phagosomal maturation and phagosome-lysosome fusion as well as modification of lysosomal contents (Koul et al.).

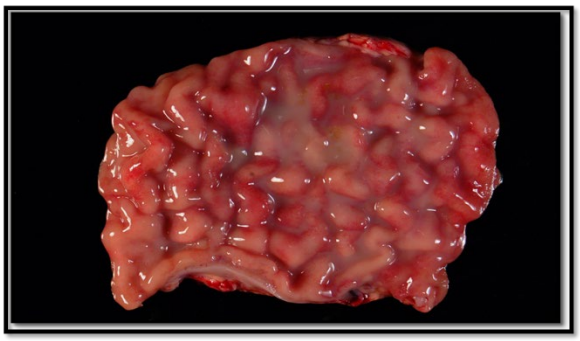
In order to survive the harsh environment presented by the macrophage, MAP secretes virulence factors to inhibit the immune response (Slavin et al, 2015). Despite all we know about MAP pathogenesis, there are still a number of crucial questions that need to be elucidated. First, the majority of MAP virulence factor remain unknown. Discovering virulence factors that MAP employs to escape death by host phagocytes is only half of the story. To fully understand MAP pathogenesis, we must understand how MAP overtakes the host cell for its own survival and replication (Paul et al.). As previously stated, MAP pathogenesis is complicated due to the slow disease progression with delayed host response. A major deficiency in clarifying the host response is a lack of *ex vivo* infection models.

Pathology

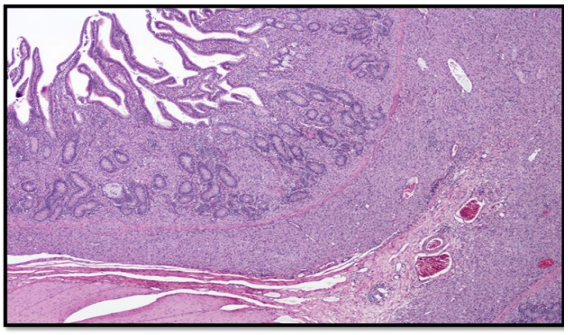
A varied assortment of pathology is observed during MAP infection. Lesions depend on the species affected, age of first exposure, and unknown individual host factors such as immune variations (L. Delgado et al.). Lesions range from no identifiable gross lesions to thick, rugose, corrugated intestine. Occasional reports of multifocal areas of ulceration and erosion of the mucosa arise; however, in the majority of cases observe smooth and shiny (nonulcerated) mucosa. In affected deer, mucosal hyperemia, erosions, and petechiation are noted (Hunnam et al.). For most species, the associated abdominal lymph nodes are enlarged and edematous (mesenteric lymphadenopathy). Lymph nodes are often calcified, especially in goats and to a lesser extent, in sheep. The lymphatic vessels are often corded, thickened, opaque dilated and visible at the gross level. Lesions are segmental and occur most commonly in the most distal portion of the small intestine but can extend proximally to the jejunum and/or distal colon in severe cases. The ileocecal valve region is usually affected.

It has been well established there is little correlation between clinical signs and lesion severity. The carcasses of animals with advanced Johne's disease are emaciated with low body condition scores. Intramedullary fat is gelatinous and there is a loss of body, pericardial, perirenal fat in these cachectic cases. Loose feces may be observed in the perineal region and covering the skin of the hind limb. Animals with intermediate disease infection have mild to moderate loss of less visceral and subcutaneous adipose tissue.

Histological lesions consist of mild to marked expansion of the mucosa, submucosa, and often the muscularis layers. Abundant foamy, epithelioid macrophages and giant cells disrupt and efface the intestinal architecture. There are scattered lymphocytes throughout the lamina propria. Within the remaining, often atrophic, crypts there is necrotic cellular debris and macrophages. The cytoplasm of the macrophages examined with an acid-fast stain contains organisms with numbers ranging from sparse to myriad. There are multifocal areas of mineralization throughout the lamina propria. Aortic mineralization, also known as arteriosclerosis, is occasionally seen with Johne's disease.



Pathology: This is a section of small intestine (ileum) demonstrating diffuse thickening of the mucosa folded into transverse rugae.
Photo by Dr. Christiane Loehr



Pathology: This is histology from the mucosal thickening shown about. There is mark expansion of all intestinal layer by due to an accumulation of macrophages with acid fast (not shown) rod shaped bacteria.

Diagnosis

Necropsy with histopathology of intestinal sections and lymph nodes is the gold standard for a conclusive diagnosis of MAP infection (Rathnaiah et al.). Poor association between clinical signs and severity of lesions adds to the complication of diagnosing Johne's disease using histology. Ziehl–Neelsen's method and microscopic examination of fresh tissues using smears from the affected mucosa and cut surfaces of lymph nodes is occasionally effective at demonstrating acid-fast organisms. Acid-fast bacteria in rectal mucosal scrapings is found to be effective in only 60% of cases (Zarei-Kordshouli et al.). However, acid-fast organisms are not visible in all cases, especially in the early stages of the disease.

Histological examination of formalin fixed tissue requires both hematoxylin-and-eosin as well as Ziehl–Neelsen-stained sections. Animals with Johne's disease can develop epithelioid macrophage microgranulomas in almost any organ. In sheep, one study found that liver biopsy core samples provided adequate tissue for histopathology with a sensitivity and specificity of 96% (Bower et al.) (Smith et al.). Full-thickness biopsies of the liver, intestines, and lymph node are diagnostic methods that could be used to provide a definitive diagnosis in particularly valuable animals.

MAP can be isolated from feces, intestinal sections, regional lymph nodes, milk/colostrum, semen, urine, fetus, and fecal tissues (Zare et al.). Fecal culture is more sensitive and specific than serology but more costly and time consuming. Culture of high traffic areas and of manure from farm sites where cattle commingle (environmental sampling) plus pooling of fecal samples can establish a herd's

infection status in a cost effective matter (Samba-Louaka et al.). Double incubation, radiometric culture, and modified Roche culture have been described recently and provide improvement in fecal culture methods (McDonald et al.)

In addition to the histologic diagnoses of MAP infections, there are many commercially available tests which have a wide range of prices, efficacies, and disadvantages. Techniques which seek to identify the organism in tissue and/or in feces via culture and PCR are the current standard of MAP testing (Stevenson). Other assays such as skin testing, interferon-γ, or ELISA for antibody to *M paratuberculosis* antigens measure the animal's inflammatory response (Scott et al.). These testing methods are commonly used to gauge the prevalence of MAP at a herd level. The dynamics of MAP infections make testing as a group, rather than a herd, more valuable in disease management. Serologic tests are rapid, low-cost, and useful to detect infection in clinically normal cattle. Within this category, ELISA tests are considered to have the highest sensitivity and specificity and are best used to determine thein determining infection prevalence in a herd (Britton et al.).

Genetic probes for IS900 insertional elements, intradermal Johnin test, lymphocyte transformation test, and interferon-γ, are generally used on a research basis for advanced clinical cases (Harris and Barletta). Complement fixation tests are available and still required by many countries for importation of animals (Kalis et al.). However, these tests are less accurate than other serologic tests. assays and therefore, rarely used less often but remain commercially available. Culture and serology tests offer greater sensitivity and specificity but are generally expensive and more time consuming for diagnosis

Control and prevention

Control measures to prevent the spread of MAP include vaccination, testing, and herd management based on producers' resources, facilities and operation (Carter). There are no cures or treatments for MAP infections resulting in JD in animals. Control and prevention measures are key to farms being free of MAP infections. Proper sanitation and management practices prevent young animals from exposure through contaminated water, feed, milk, or colostrum (R. J. Whittington et al.). Neonates should be bottle-fed pasteurized colostrum (Stabel). Milk and colostrum obtained from dams that test negative may be used as well (Stabel). Importantly, routine testing of managers and their environmental should be performed in order to identify and any terminally remove positive animals. Many States offer Johne's certification to test-negative herds. Purchase of replacement animals from certified negative herds should be strictly scrutinized.

Although there are JD vaccines that reduce clinical disease and shedding, they all have limitations in providing long-term robust immunity. Vaccination of calves <1 month old can reduce disease incidence but won't prevent shedding or new cases of infection in the herd (Bannantine and Talaat). As vaccination won't eliminate MAP, good management and sanitation are just as important to herd health. Vaccination is ultimately beneficial as herds vaccinated for MAP have increased milk and meat output (Shippy et al.). There is a need for a more effective, affordable, and safe vaccination which prevents infections. Cattle injected with inactivated whole-cell, mineral-oil product may cause based vaccines can develop granulomas (R. Whittington et al.). The lack of an effective vaccine and an accurate method of

detection are major hurdles hampering the goal of eliminating MAP infections. To develop a proper vaccine and methods for control and prevention, we must first understand the mechanisms by which MAP subverts the host immune responses.

Zoonotic Potential of MAP infection

MAP has a broad host range and has been isolated from domestic and wildlife ruminants, including beef and dairy cattle, sheep, goat, antelope, deer, bison, and many other species. There is a growing concern that MAP from dairy cattle may also present a threat to humans. Zoonotic pathogens have long been associated with the human food production systems. These pathogens have required major public health intervention to prevent and reduce illness and death in people. The notion of MAP as a food and food production systems-associated zoonotic pathogen is gaining momentum. The zoonotic role of MAP in Crohn's disease, ulcerative colitis, and other forms of immune-mediated enterocolitis of humans has been investigated for several decades. Despite considerable research, evidence to support MAP as a significant cause of human disease remains inconclusive. Although higher levels of MAP are found in the tissues and blood of human CD patients compared to controls, the bacteria are often challenging to detect in patients presenting with clinical signs (Hruska et al.). This may be due to MAP being incredibly slow-growing and difficult to culture. Additionally, the amount of shedding is intermittent, and thus the so infection may not be confirmed detected during a routine solitary culture. In one study, MAP was detected via PCR in the gut of 92% of patients with Crohn's disease (Davis et al.). This leads us to believe that the true prevalence of MAP may be higher than reported among CD patients.

CD is a chronic inflammatory condition with symptoms consisting of mild to moderate diarrhea, abdominal pain and fever. In severe cases, protein losing enteropathy, vitamin deficiency, and bowel perforation may occur (Waddell et al.)(Davis et al.).There are two main hypotheses about the role of MAP in relationship to CD patients. Firstly, it is hypothesized that MAP is one of the main causes of CD. Lesions observed during histological examination as well as culture and PCR results support this speculation. The second theory is that MAP prevalence is due to CD-attributed immune dysfunction. (Naser et al.). People genetically predisposed to develop CD have increased levels of MAP positive cultures, suggesting MAP might be one of the etiologic agents involved in the development of CD (Zarei-Kordshouli et al.) (Ellingson et al.). If this is true, public health interventions are needed to prevent necessary in preventing MAP contamination into the food supply.

MAP cells are able to travel through the mammary glands into milk products for humans because it's resistant to pasteurization (Zarei-Kordshouli et al.). In addition to withstanding a variety of environmental conditions, the organism survives high-temperature, short-time pasteurization (72°C for 15 seconds) if present in sufficient numbers in raw milk (Patel and Shah). MAP survives the process of pasteurization due to its thick, waxy cell wall. Epidemiological studies mapping cases of Crohn's disease show unusually high numbers of cases in areas near rivers which drain agricultural farmland (Waddell et al.). MAP has also been recovered from breastmilk samples of human patients which may be a means of infection in young humans. Further research is needed to fully elucidate the role playedMAP plays, if

any, in the disease progression of CD, ulcerative colitis, and other inflammatory bowel conditions.

Scope of Doctoral Dissertation

The aims of the following dissertation are to examine MAP-host interactions utilizing existing *in vitro* models. The overall long-term goal is to unveil the molecular mechanisms of how MAP establishes persistent infection within the host and to identify host cellular responses that promote MAP survival to contribute to the development of Johne's disease. We examined genes involved in alterations to the host response and the host response itself. We hypothesized that immune alteration of the infected host is caused by a discrete set of virulence factors and the global immune response and outcome depend on the movement of the bacteria through the host. *Acanthamoeba castellanii* (amoeba) served as a rapid screening protozoan model for phagocytic cell infection and survival. Mycobacterial pathogens have been demonstrated to acquire survival strategies within protozoan hosts in the environment (Tenant et al, Harriff et al) and we hypothesized that by evaluating changes in amoeba metabolism during MAP infection, we could identify bacterial virulence genes that are important for macrophage infection and survival.

Additionally, to identify host phenotypes and cellular immune responses that benefit MAP persistence and spread between epithelial cells and macrophages, we used an *in vitro* cell culture passage model which mimics the intestinal mucosa. Our hypothesis was that during infection of different cell types, bacteria develop distinct cellular phenotypes (by modifying metabolism and cell surface factors) and these phenotypes promote host cellular responses that enhance MAP survival. Investigation

of bacterial virulence factors and cellular responses expands our understanding of the of phagocytic cell signaling pathways and immune responses allowing pathogen survival within the host. Identifying MAP virulence factors involved in host persistence and determining phagocytic signaling pathways during MAP infection facilitates strategies for intervention. Ultimately, the work described here enhances the field of MAP research, and the tools available to study the disease. Our studies expand upon the phenotypic changes acquired by MAP and the role of those changes upon host epithelial cell infection. These changes can be monopolized when designing new prevention strategies for MAP infection. The following data adds novel observations and findings to the field of MAP and Johne's disease research while providing new models and systems for future investigations of bacterial virulence mechanisms. Elucidating the MAP-host interface provides the foundation for enhanced management options to control MAP infection in ruminant populations worldwide.

Chapter 2

High Throughput Screening of *Mycobacterium avium* subspecies *paratuberculosis* library using *Acanthamoeba castellanii* (amoeba) as a host model for host macrophages

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Infection and Immunity: Manuscript in Preparation

Abstract

The high prevalence of Johne's disease has driven a continuous effort to understand the pathogenesis of the bacterium and develop more effective preventative measures curbing disease spread within herds. In this study, we aimed to create a quick and more effective model for studying *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection. We utilized *Acanthamoeba castellanii* (amoeba) as a phagocytic host to understand the interaction between MAP and the environmental phagocytic protozoan during infection. Using the Alamarblue assay, we demonstrated that MAP stimulated the metabolism of the amoeba, and that stimulation . These changes directly mirrored the pattern of survival and the MAP intracellular burden over the course of infection. We identified bacterial mutants withexcessive or deficient metabolic activity stimulation within host cells. Our studies support the use of model systems for studying Johne's disease and MAP infection and in order to further our understanding of the host-pathogen interactions. Analyzing MAP virulence factors unveils the complex strategies used by the pathogen in our quest to facilitate the detection, diagnosis, and prevention of the disease. These findings offer novel methods for the identification of new bacterial phenotype targets that could be used as the basis of developing more efficacious strategies in detecting and preventing Johne's disease.

Introduction

Ruminants worldwide developed progressive granulomatous enteritis after becoming infected by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Neonatal calves ingest the bacterium in feces found in contaminated colostrum, milk, feed, or water but do not develop disease for several years (Aaron et al.). Johne's disease is often categorized into 4 stages: silent infection, subclinical infection, clinical, and advanced clinical disease (Berry et al.)(Wu et al.). Stages are defined by the severity of clinical signs and the potential shedding of organisms into the environment. One of the difficulties in controlling MAP infection is that animals frequently shed MAP in feces prior to showing any clinical signs. This is a contributing factor in spreading the infection to other animals in the herd.

In cattle, Johne's disease slowly progresses as the bacterium divides inside host macrophages while avoiding host defense mechanism. Macrophages are the preferred host cell for MAP, therefore, the ability of pathogenic mycobacteria to undermine these mechanisms is essential for survival and persistence. It is still unknown how MAP overcomes the cell's well-coordinated and effective Despite what is known about MAP infection, there are remains unanswered basic questions unanswered. For example, most of the virulence factors for MAP are still unknown. In this study, we developed *in vitro* models to discover possible MAP virulence factors. *In vitro* modeling of MAP infection allows us to study MAP pathogenesis without the high costs, time, and variability associated with large animal studies.

Free-living amoeba (FLA) have been frequently isolated from habitats common to mycobacteria (Thomas et al.) (Ovrutsky et al.). These organisms interact

with environmental bacteria-like members of the mycobacterial groups. These findings strongly support the notion of an "endosymbiotic" relationship between mycobacteria and the host FLA. It is believed that amoebal cysts protect intracellular mycobacteria against adverse conditions and serve as a vector for dissemination ((Ben Salah and Drancourt) (Iovieno et al.) (Vollmer and Glaser). MAP and other mycobacterial agents avoid FLA predation using the same strategies to resist macrophage destruction

Mycobacterium avium subspecies *hominissuis* (MAH) is pathogen of immunosuppressed humans with more than 90% homology to MAP . Our lab found that this bacterium can infect environmental amoeba and when it does, it acquires a more virulent phenotype upon re-isolation from *Acanthamoeba castellanii* in the mammalian host (Tenant and Bermudez). We also found that gene expression in MAH infected amoeba was found to be similar to those in MAH infected mammalian macrophages. Another study found that mutants with a defect in the ability to invade mammalian macrophages were also deficient in amoeba invasion. These studies support the hypothesis that mycobacterial species have evolved mechanisms that are used within the amoeba and for survival within macrophages.

Our study uses the amoeba *Acanthamoeba castellanii* to screen a library of MycomarT7 with the Himar promotor transposon MAP mutants. that MAP infection induces a dramatic stimulation of the metabolic activity of the amoeba. We show that the increased activity is MAP-dependent and differences in metabolic activity can be seen when the mutant bacteria are used. We can eliminate mutants with metabolisms which are the same as WT and more closely examine those with deviations from WT.

We confirm the amoeba model using. Amoeba models were validated against Raw 264.7 macrophages.

Methods and Materials

Bacterial strains and cultures ATCC BAA-968 strain K10 of *Mycobacterium avium subsp. paratuberculosis* was used for all studies described in this chapter. The bacterium was cultured at 37°C on 7H10 agar enriched with 1 g of casein hydrolysate, 100 mL of oleic acid, albumin, dextrose, and catalase (OADC) and 2 mL ferricmycobactin J for 4-6 weeks. Inoculums were prepared by adding bacteria to 5mL of phosphate buffered saline (PBS). The bacterial suspension was allowed to settle for 10 min then the top 2/3 was aliquoted in a 14 mL polystyrene tube. The mixtures underwent passage through a 1.5 inch 22-gauge needle 15 times to disrupt clumps that may be present. The suspension was adjusted to match McFarland standard.

Amoeba culture *Acanthamoeba castellanii* (ATCC 30234) were cultured in a T-25 flask in 712 Peptone-Yeast-Glucose (PYG) (10 g proteose peptone, 0.5 g yeast extract, 4 mM magnesium sulfate heptahydrate, 400 μM calcium chloride, 3.4 mM calcium chloride, 50 μM ferric ammonium sulfate hexahydrate, 2.5 mM disodium phosphate heptahydrate, 2.5 mM potassium phosphate, 0.1 M glucose, pH 6.5) media. The organisms were cultured in a dark 25°C incubator overnight. The cells were then scraped and placed in a T-75 flask and allowed to grow for one week with intermittent media changes.

Mammalian cell culture.

RAW 264.7 macrophage were purchased from American Type Culture Collection's (ATCC; Manassas, VA) and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products; West Sacramento, CA), at 37°C in 5% CO_2. Cells seeded on the T-75 flask were dislodged with a cell scraper and the appropriate aliquots of the cell suspension was added into new culture vessels.

MycomarT7 transduction and construction of MAP transposon library (Transposon Mutagenesis)

The temperature sensitive transposon-containing phagemid MycomarT7 (Mmt7) (Sassetti et al.) was used to create the MAP transposon bank of mutants as reported previously (Rose and Bermudez). φMycoMarT7 includes a transposon that encodes a kanamycin resistance gene and T7 promoters which promotes transcription of chromosomal DNA. The entire sequence of the MycoMarT7 transposon has been deposited in GenBank (GenBank accession no. AF411123). The phagemid also contains the highly active C9 *Himar1* transposase gene. For transduction, 100 mL of MAP cultures were grown to OD_{600} in Middlebrook 7H9 broth with OADC (10% v/v), mycobactin J (2 mg/ml), and kanamycin sulfate liquid culture. Cells were washed twice with a mycobacterial buffer solution (50 mM Tris, pH 7.5/150 mM NaCl/10 mM $MgSO_4$/2 mM $CaCl_2$) and resuspended in the buffer solution. Bacterial cells were infected with 10^{10} phage for 3 hours at 37°C. Transduced cells were plated directly Middlebrook 7H10 agar with OADC (10% v/v), mycobactin J (2 mg/ml), and kanamycin sulfate at 37°C cultured at 37°C. After transduction, individual clones were selected and grown in 300ul of Middlebrook 7H9 broth with OADC (10% v/v), mycobactin J (2 mg/ml), and kanamycin sulfate at 37°C in 96 well plates. The

kanamycin-resistant colonies were replated and screened for the presence of Mmt7 with PCR twice.

Mmt7 Gene Knockout Clones location identification

Transposon insertion locations on mutants of interest were identified utilizing a previously reported ligation-mediated PCR (LMPCR) technique, with minor modifications as previously reported ((Patel et al.), (Prod'hom et al.)).

The final PCR products were run on agarose gel electrophoresis to be visualized using ethidium bromide. To obtain bacterial genomic DNA, approximately 1.5 mL of turbid suspension from each MAP mutant was lysed, cleaned, and concentrated using the Zymo DNA Clean and Concentrate kit according to kit protocols (Zymo Research Irvine, CA). The purified DNA were digested using Sall restriction enzyme. The digested genomic DNA was used to create a ligation reaction. For the ligation reaction, two different adapter oligos were created using salpt and salgd and separately bampt + salgd, 4.5uL of each oligo, and 1 uL of magnesium chloride. Over the course of 1 hour, the temperature was gradually reduced from 80˚C to 4˚C using the Bio-Rad T100 thermal cycler. The ligation-mediated PCR reaction used the ligated product for the PCR template. The PCR reaction ran as initial denaturation at 95˚C for 5 minutes followed by 38 cycles of 95˚C for 30 seconds, 58˚C for 30 seconds and 72˚C for 1 minute 45 seconds.

The PCR products were visualized by agarose gel electrophoresis. Bands that were l50-bp apart were excised, gel purified, and submitted to the Center for Genome Research and Biocomputing at Oregon State University for DNA sequencing.

Bacterial Infection of Amoeba. Amoeba seeded onto glass chamber slides were infected with MAP at an MOI of 10:1 in 712 PYG media and incubated for 1 hour at room temperature (25°C). Slides were washed 3 times with PBS to remove extracellular bacteria, gently heat-fixed, and stained using Kinyoun's acid fast protocol to identify intracellular mycobacteria (Kinyoun 1915). Slides were visualized on a DM4000B Leica microscope under bright field and fluorescent conditions. Images were captured and analyzed using QCapture Pro7 software (QImaging; Surrey, BC, Canada).

Establishing amoeba metabolic activity assay. 96-well tissue-culture plates were seeded with amoeba overnight in 712 PYG. Cells were infected with the following samples in 712 PYG: media only, MAP (MOI 100:1, 50:1, 10:1, 1:1). Infections were synchronized by centrifugation at 225 × g at 25°C for 5 minutes and incubated for 2 hours at 25°C in the dark. Following the infection, wells were washed 2 times with Page's Amoeba Saline. Fresh 712 PYG media supplemented with 10% (v/v) AlamarBlue (Life Technologies) was added to each well. Fluorescent readings of each plate were measured at 530nm/590nm (excitation/emission) every hour for 24 hours using a Tecan F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). For long-term experiments, amoeba were infected as described above, synchronized, washed with Page's amoeba saline, and fresh 712 PYG media was added to each well. At time 0, and every 12 hours until 180 hours post-infection, AlamarBlue (10% v/v) was added to a new set of infected wells and fluorescent readings were taken at 0, 2, 12, and 24 hours after the addition of dye.

Metabolic screening of MAP mutant library using Alamarblue. 96-well tissue-culture plates were seeded with *Acanthamoeba castellanii* (ATCC 30234) for two hours in 712 PYG. After the two-hour period, the amoeba were infected with WT MAP or transposons mutants at a MOI of 50:1. Control wells included media only, uninfected amoeba, and WT infected cells. The infections were synchronized by centrifugation at 250 RPM at 25°C for 5 minutes. The plates were then incubated for 2 hours at 25°C in the dark. This was followed by three Hank's buffered salt solution (HBSS) washes. 300 uL of 712 PYG media supplemented with 10% (v/v) AlamarBlue (Life Technologies) was added to each well. Fluorescent readings of each plate were measured at 530nm/590nm (excitation/emission) every hour for 24 hours using a Tecan F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Mutants that increased amoeba metabolism (2.5 fold change) were selected for sequencing. In total ~2500 individual clones were analyzed and the clones with the greatest change of metabolic stimulation from the wild-type infection were selected for further analysis.

Macrophage Invasion and Survival Assays. Monolayers of Raw 264.7 seeded in 24 well plates overnight in DMEM were infected with MAP or transposons mutant at a MOI of 10 bacteria (10 bacteria per cell). The plate was spun to synchronize the infection at 250 RPM for 5 minutes and incubated at 37°C for 2 hours. The supernatant was removed and the wells were washed three times with Hank's buffered salt solution (HBSS) or PBS. DMEM media with Amikacin (200 g/ml) was added for 2 hours at 37°C to kill extracellular bacteria. Afterwards, the monolayer was washed three times and lysed with 500uL of 1% Triton X-100 in HBSS for 15

min. 100 uL of lysate was serially diluted and plated for CFU determination onto 7H10 agar enriched with 1 gram casein hydrolysate, 100 mL of oleic acid, albumin, dextrose, catalase (OADC) and 2 mL ferric mycobactin J. The cells were lysed and the bacteria were plated for the other time points (24 and 96 hours).

Complementation and Macrophage Assays The four selected MAP mutants 18D6: MAP_2291, 7C12: MAP_0949, 15G2: MAP_3634, and 3D3: MAP_3893c were selected for complementation. The mutant bacteria were grown in 100 mL Middlebrooks 7H9 media for four weeks. The cultured bacteria were centrifuged and washed four times in 25 mL deionized washing solution containing 10% glycerol and 0.1% TweenTween-80. Cells were electroporated with 7 μg of the plasmid pMV306 constructs. The bacterial cells were cultured in 5 mL Middlebrook 7H9 broth, OADC (10% v/v), and mycobactin J (2 mg/ml) at 37°C for 24 hours. They were then plated on 7H10 media with OADC (10% v/v), and mycobactin J (2 mg/ml) at 37°C for 8-12 weeks. Macrophage survival assays were repeated using the complemented strains as described before.

Statistical analysis and data interpretation. Results are reported as the mean of at least 2 independent experiments ± standard error. For survival complementation assays, statistical comparisons between experimental groups and control groups were determined using the Student's *t*- test with $p<0.05$ denoting statistical significance. Survival curve data was analyzed using Kaplan Meier Survival Analysis. GraphPad Prism version 6.0 software was used for the construction of graphs, data interpretation, and all statistical analyses.

Results

MAP stimulates the metabolism of *Acanthamoeba castellanii* amoeba during infection

Previously in our lab, cultures were infected and both extra-and intracellular bacterial viability was monitored over a 15-day course of infection to deduce the long-term conclusion and effects of MAP infection on amoeba and determine the viability of intracellular MAP over the course of infection. The summary of those results provided the foundation for the metabolic activation assay performed in this study. The previous results found that within 24 hours of infection, the amoeba eliminates almost 50% of MAP. The amoeba can control the intracellular bacterial burden until 3 days post-infection. At the 72-hour mark, the intracellular population begins to replicate. By day 5, the bacterial levels are restored to the initial infection level. To determine the impact that MAP infection had on the overall metabolism of the amoeba, a metabolic activity assay was conducted using the dye AlamarBlue as an indicator of oxidative respiration. The intensity of the fluorescence corresponds to the metabolic activity of the amoeba. The measured fluorescence values demonstrated that MAP infection increased metabolic activity of the amoeba over the first 24 hours of infection (Figure 2). The increased metabolism was dose-dependent as an MOI of 10 resulted in a much higher metabolic stimulation compared to that at an MOI of 1 (data not shown), though both metabolism levels are significantly higher than uninfected amoeba. Since rapidly growing bacteria can result in a shift in AlamarBlue fluorescence (Rampersad), the metabolic activity of the bacteria during

growth was also examined. In the absence of amoeba, MAP alone results in minimal fluorescence over the course of the assay (Figure 2).

To determine whether the bacterial-induced increasing of amoebic metabolism was due to live MAP or simply stimulated by the cellular components of MAP alone, amoeba were infected with heat-killed bacteria for an equivalent duration of time. Neither the heat-killed bacteria nor the heat-killed MAP infected amoeba resulted in any increase of phagocyte metabolic activity (unpublished data). *Acanthamoeba castellanii* has been described to release an oxidative burst upon phagocytosis of inert beads (Wiesner). To confirm that the metabolic stimulation observed in this assay was due specifically to the ingestion of the pathogen and not a bystander effect, phagocytosis was assayed with 0.2 μm latex beads. Upon uptake of latex beads, we observed negligible metabolic activity stimulation in the amoeba during the course of the assay (unpublished data). Therefore, these data demonstrated that the metabolism of amoeba is highly upregulated by the ingestion of MAP during infection and the metabolic increase is dose-, viability-, and particle-dependent.

Identification of MAP transposon mutants with amoebic metabolic activity assay

Amoeba as protozoan organisms have similar defense mechanisms as mammalian macrophages. These strategies are successful at killing many bacterial, fungal, and protozoal agents that the amoeba contact within the environment. Our previous data found that 50% of the MAP entering the amoebic host is eliminated within 24 hours which is correlated to concurrent metabolic hyperstimulation. As stated above, after 72 hours, MAP begins to grow and are back to regains original levels by day five. MAP can overcome the amoeba's defenses to grow inside the host.

We hypothesized that one or more virulent components of the bacterium are responsible for the survival and persistence within the protozoan, similar to those MAP uses to survive in ruminant host cells. To determine which bacterial components are responsible for persistence, a transposon library of MAP was constructed and screened with the AlamarBlue metabolic assay to identify mutants with the highest change in amoeba metabolic stimulation during 24 hours of infection (Figure 2). Each 96 well plate contained wild-type MAP infected amoeba which served as an internal control and comparison for amoebic metabolic stimulation. This helped account for minor variations in the protocol including changes in room temperature and alteration of timepoints collected over the 24 hour infection. We identified that the M.O.I. of MAP can affect the metabolism levels of amoeba (data not shown). Therefore, the bacterial density of each culture was measured and the infection level adjusted to ensure the M.O.I. was not responsible for increased or decreased metabolic activity within the amoeba. Of the roughly 30 plates that were screened, the highest and the lowest metabolic curves were identified. The MAP mutant strain used for infection of those respective wells was re-cultured on 7H10 agar plates (Figure 3). The MAP mutants were then divided into four main groups by fold change in metabolism (Figure 3). There were 573 mutants at 1-1.5-fold below wild-type and 501 mutants above 1-.1.5. For the 1.5-2 group there was 214 below wild-type stimulation and 207 above wild-type metabolism. Forty mutants caused lower causing decreased activation than wild-type and 53 mutants causing more amoebic causing increased amoeba activation than wild-type were in the 2-2.5 fold

change group. Finally, MAP mutant numbers at 2.5 fold higher or lower were 28 and 17 respectively.

Each selected clone was re-analyzed twice for metabolic activity during amoeba infection. Strains that did not produce consistent results over 3 independent experiments were eliminated and the mutant pool of interest was narrowed to 40 mutants (Figure and verification data not shown).

Macrophage validation assays

To confirm mutants identified above, we analyzed the virulence of each strain using murine macrophages Raw 264.7. Each selected mutant was assayed for its level of uptake and its ability to survive at 24 and 96-hours post-infection (Figure 4a and 4b). The amount of bacteria which entered the macrophages averaged 2% for both wild-type and mutant strains (data not shown). To determine if the strains had differential differed in their survival, assays were conducted in which upon uptake, intracellular MAP were quantified and normalized to their respective uptake values to account for the differences in initial bacteria present within the cells. Notably, at 24-hours post infection, most of the strains that stimulated increased metabolic activity of the amoeba exhibited equivalent or increased intracellular survival rate compared to wild-type MAP infection (Figure 4a). In contrast, many of the mutants that failed to increase amoeba metabolism demonstrated lower survival rates within the Raw 264.7 cultures after 24 hours of infection (Figure 4b). When analyzed for intracellular survival within Raw 264.7 after 96 hours of infection, all tested strains were able to resume growth, replicate within the cell, and maintain a steady rate of infection over the long-term. Thus, these mutants identified using amoeba as the host phagocyte also

survived in murine macrophage cells, verifying the validity of our findings using the amoeba model.

Isolation of MAP Mmt7 Transposon Mutants (Mutant sequencing)

The molecular mechanisms of each mutant were under covered by isolation of the transposon. Table 2 lists the mutant names, response to the Alamarblue assay, survival assay results, the gene name and domains within the affected gene. We sequenced the 40 mutants that caused a 2.5-fold increase in metabolism over wild type MAP. Ligation-mediated polymerase chain reaction (LM-PCR) is a genomic analysis technique for determination of primary DNA nucleotide sequences. Once purified DNA was obtained, we submitted it to the Center for Genome Research and Biocomputing at Oregon State University for sequencing. Based on NCBI and Kyoto Encyclopedia of Genes and Genomes database. Clone 18F6 (MAP_2291), clone 7C12 (MAP_0949), clone 15G2 (MAP_3634), and clone 3D3 (MAP_3893c) were selected for complementation. They were selected because they were found to be attenuated in the macrophage assay, have domains associated with pathogen virulence, or have been associated with survival in other mycobacterial species. National Center of Biotechnology Information databases were used to analyze the genomic sequences interrupted by the bacteriophage transposons. Mutants included: MAP_2291 (truncated hemoglobin which is used to protect pathogens against oxidative damage (Pawaria et al.)), MAP_3893c (protein kinase shown to block phagolysosome fusion (Bach et al.)), MAP_0949 (second messenger system for regulation of oxidative adaptation (Gomelsky and Galperin)) and MAP_3634 (IgD-

like L transpeptidase which have been associated with virulence and resistance in M. tuberculosis (Mtb) (Erdemli et al.)).

Complementation restores WT phenotype

To examine whether the replacement of the four selected MAP mutants 18D6: MAP_2291, 7C12: MAP_0949, 15G2: MAP_3634, and 3D3: MAP_3893c genes was associated with reversion of the attenuated phenotype, Raw 264.7 cells were infected with the complemented mutant containing a copy of the various genes (Figure 5). Increased growth was seen in the complemented strains of all four genes. This result suggested that the inactivation of these genes decreased survival within the host.

Discussion

MAP is a pathogen which uses a variety of methods to manipulate the host defenses and metabolic state. Usually, infections trigger an innate response which serves to eliminate the pathogen. However, MAP overcomes host defenses by subverting killing and increasing bacterial access to pro-survival compounds or nutrients within the phagocytic vacuole. Metabolic shifts induced by pathogens are sometimes difficult to unravel in the context of the host-pathogen interaction. The interpretation of such data can be complex if one considers other variables such as host cell and chosen time points for analysis.

Several mammalian macrophage cell lines have been used to study the immune responses to MAP. *Acanthamoeba castellanii* infection results in a similar pattern of upregulated genes as *Mycobacterium avium* seen during macrophage infection ((Danelishvili et al.). Eukaryotic organisms, like amoeba, have long been noted to interact with mycobacterial species in the environment (Ovrutsky et al.). Our lab has found that MAP and other mycobacterial agents can infect amoeba (Danelishvili et al.). MAP is closely related to *Mycobacterium avium* subspecies *hominissuis* (MAH) which also infects environmental amoeba and becomes more virulent upon isolation (Tenant and Bermudez).

Macrophages are evolved from metazoan phylogeny, many of which still exists in nature today. Other studies have shown that *M. avium* have evolved mechanisms to survive within amoeba with the host protozoa protecting phagocytized mycobacteria from extreme temperature, drought and diverse biocide attacks via cyst formation (Barker and Brown,; Ben Salah and Drancourt; Denoncourt et al.,). Growth

of *Legionella pneumophila* in multiple amoeba species is from selection/acquiring of various virulence genes that also promote bacterial survival in human macrophages (Ovrutsky et al.). The bacteria are able to make use of protozoan nutrients and prevent degradation. Also, prolonged interactions between pathogens and hosts result in selection for traits that increase virulence or survival in the host (Iovieno et al.). For opportunistic pathogens acquired from the environment, these traits must have evolved for increased fitness. It is theorized that protozoa environmental interactions are a driving force for the evolution of virulence in opportunistic pathogens (Thomas et al.).

Our studies found that MAP survival is adversely affected during high metabolic activity within the amoeba host while a slow metabolic state results in growth and enhanced bacterial survival. Over the same time period, the metabolism of infected amoeba is significantly higher than that of uninfected amoeba. Aligning the data from the screening assay with that from the macrophage survival validation assay, we find a mild correlation between hyper-stimulatory mutants and survival after 24 hours within the murine macrophage. Many of the mutants attenuated after 24 hours, conversely, produced hour had metabolic activity much lower than WT. It is important to note that after 96 hours of infection, most of the mutants were attenuated. Overall, these assays confirmed the findings that the attenuated mutants identified in amoeba screen are, in fact,attenuated within Raw 264.7 macrophage cells.

These bacterial factors, although not true homologues to corresponding *Mycobacterium tuberculosis* proteins, have been shown to contribute to *M.*

tuberculosis survival in the host. The complementation assay reveals that activation of MAP_3893c (serine/threonine- protein kinase PknG) leads to activation of virulence. Protein kinase G (PknG) is a virulence factor in *Mycobacterium tuberculosis* and a thioredoxin-fold–containing eukaryotic-like serine/threonine protein kinase (Xu et al.). It is well known and researched that this kinase is required for inhibition of phagolysosomal fusion (Walburger et al.). Recently, it was unraveled that this protein plays a role in Mtb during latency-like conditions by mediating persistence under stressful conditions like hypoxia. PknG also promotes drug tolerance (Khan et al.). PknG protein has been implicated in various macrophage functions like phagocytosis, maturation of phagosome, immunity to infection, apoptosis, and the production of cytokines/chemokines/immune effector molecules (Schaible et al.) (Kidwai et al.). The exact mechanisms of action of this protein are not well understood during MAP infection. How PknG is secreted and released into the cytoplasm of bacteria-infected macrophages remains unknown. It is our hypothesis that bacterial PknG binds to a host protein which is involved in phagosome formation, maturation, trafficking, or lysosomal fusion. These proteins include but are not limited to: soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) protein, small Rab GTPase (Rab) proteins, and vesicle associated membrane protein (VAMP) proteins. In Mtb, PknG target Rab protein Rab711 to prevent the formation of the phagolysosome (Pradhan et al.) MAP_RS11665, formerly MAP_2291, is a protein coding globulin which is found across the *Mycobacterium avium* complex (MAC) and other subspecies of MAP including (MAP4, CLIJ623, Pt139). Importantly, in *Mycobacterium tuberculosis*

(Mtb) glbN gene encodes the truncated hemoglobin N (trHbN)[4] (Ascenzi and Visca) (Yh et al.). Mtb trHbN is associated with nitric oxide (NO) detoxification and oxidative stress reduction in response to macrophage nitrosative stress (Daigle et al.). This family of proteins have unique and diverse sequence properties but all share a function related to oxygen affinity and reactivity including O_2, NO, and CO interactions (Bustamante et al.). The domains present include TrHb2_Mt-trHbO-like_O; Truncated hemoglobins, group 2 (O); and Mtb hemoglobin O like domain. Our macrophage survival assay with this mutant found that it was deficient in uptake and survival over a 96-hour time point.

The two-component signal transduction (TCS) machinery is a key mechanism of sensing environmental changes in the prokaryotic world (Mitrophanov and Groisman). TCS systems have been characterized thoroughly in bacteria but to a much lesser extent in archaea (Mitrophanov and Groisman). They influence bacterial cell survival, biofilm formation, virulence, and bacteria–host interactions (Li et al.). However, many of their cellular targets and biological effects are yet to be determined. Signaling nucleotides like c-di-GMP, cAMP, and ATP are second messengers which respond to and control the bacterial expression of a variety of environmental and cellular signals through amino-terminal sensory domains (Wei et al.). MAP_RS04805 (MAP_0949) is a 1,854 nucleotide EAL domain-containing protein. This mutant is severely attenuated in host macrophages; therefore, this protein likely endows MAP with a molecular strategy important to survival within a host. The gene has not been previously characterized in MAP. Nearly all bacteria

have GGDEF, EAL, and HD-GYP domain proteins, with many encoding multiples of these enzymes.

MAP_3634 gene is a L,D-transpeptidase which is responsible for the final polymerization steps involved in the formation of the glycan strands and cross-linking of peptide stems of the peptidoglycan cell wall of many bacteria (Mainardi et al.). L,D-transpeptidases has been identified in numerous bacteria, including *Enterococcus faecium*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Clostridium difficile*, and *E. coli* (Schoonmaker et al.). Inactivation of these transpeptidases has been shown to be bactericidal in Mtb and *Mycobacterium abscessus* ((Dubée et al.) (Kumar et al.)). Carbapenems and amoxicillin antibiotics have been characterized as responsible for inhibition of L,D-transpeptidases of *Mycobacterium tuberculosis* and a range of bacteria including ESKAPE pathogens (Kumar et al.). We hypothesize that MAP_3634 mutant is killed within the macrophage due to defects within the cell wall structure. Protein secretion mechanisms and transport pathways to the *trans* side of the peptidoglycan requires functional L,D-transpeptidases (Geiger et al.). Loss of function for this protein may lead to other virulence factors being unable to leave the bacterial cytoplasm resulting in bacterial death.

In total, these data indicate that MAP has a dramatic impact and effect on the metabolic activity of the host phagocyte during infection. Metabolic changes correlate with the intracellular bacterial burden of MAP over the course of infection. Increased metabolism appears related to decreased bacterial viability while a slower, less dramatic metabolic change corresponds to replication within the phagocyte. We

confirm that the use of amoeba is a powerful model in discovering aspects of the pathogenic genome which contribute to disease.

Primer Name	**Sequence**
MAP_0949	
Forward	TTTTTAAGCTTgtgccacgcagcctggac
Reverse	TTTTTCGCCGGCGcatcgacgacgcctcgc
MAP_2291	
Forward	TTTTTAAGCTTatggatcaggtgagcttc
Reverse	TTTTTCGCCGGCGtcacaacggagaattcac
Map_3634	
Forward	TTTTTAAGCTTAtgagtggaggtatgcct
Reverse	TTTTTCGCCGGCGCTAGTTCATCCAGTCGGC
Map_3893c	
Forward	TTTTTGGATCCATGGCCGAGCCGGA
Reverse	TTTTTGAATTCTCAGAACGTGCTGGTGGG

Table 1 Primers used in complementation of the MAP transposon mutants

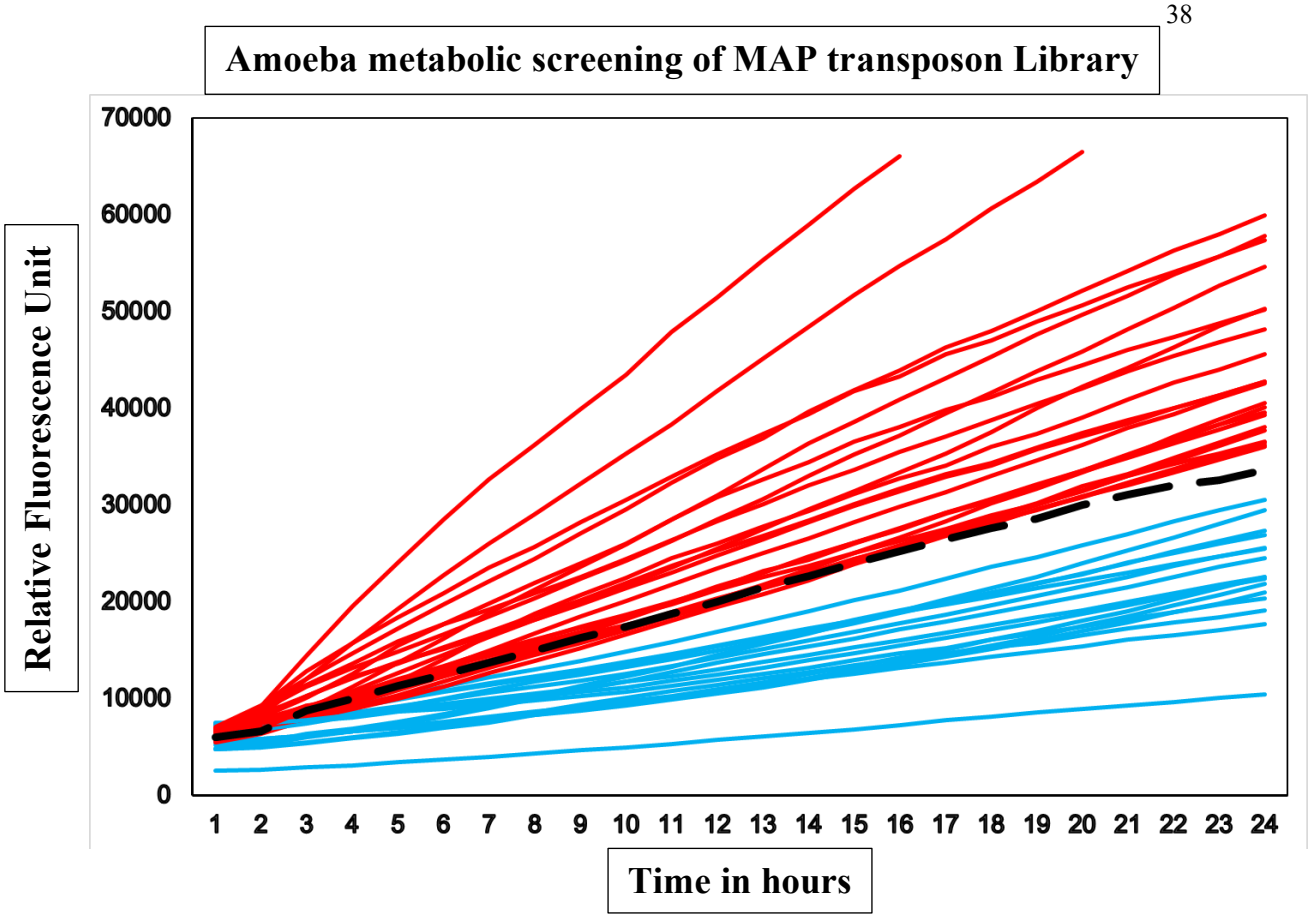

Figure 2 MAP transposon library screen results. The dashed line in black represents the metabolism of the amoeba induced by wild-type MAP infection at MOI of 10:1 over a 24 hour period. Mutants shown here are those which produced metabolism at 2.5 times greater than (red line) or less than (blue line) that produced by WT MAP.

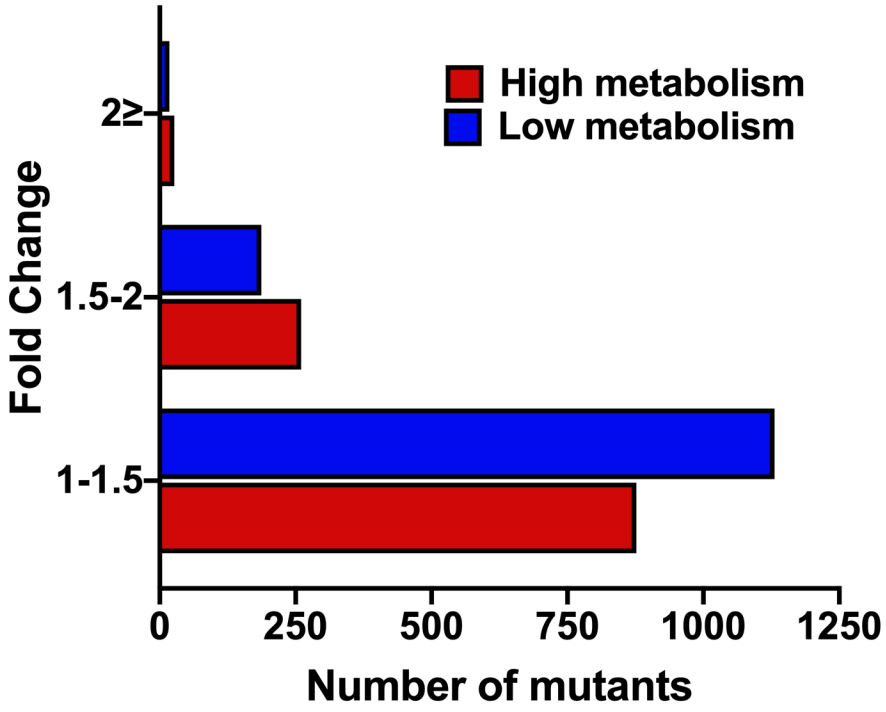

Figure 3 Results of the MAP mutant library screen. The majority of the mutants caused a change in amoeba metabolism that was similar to that caused by wild type infection. We found 45 mutants that caused a change in the amoeba metabolism 2.5 fold higher or lower than wild type. These mutants moved forward to the next experimental projects.

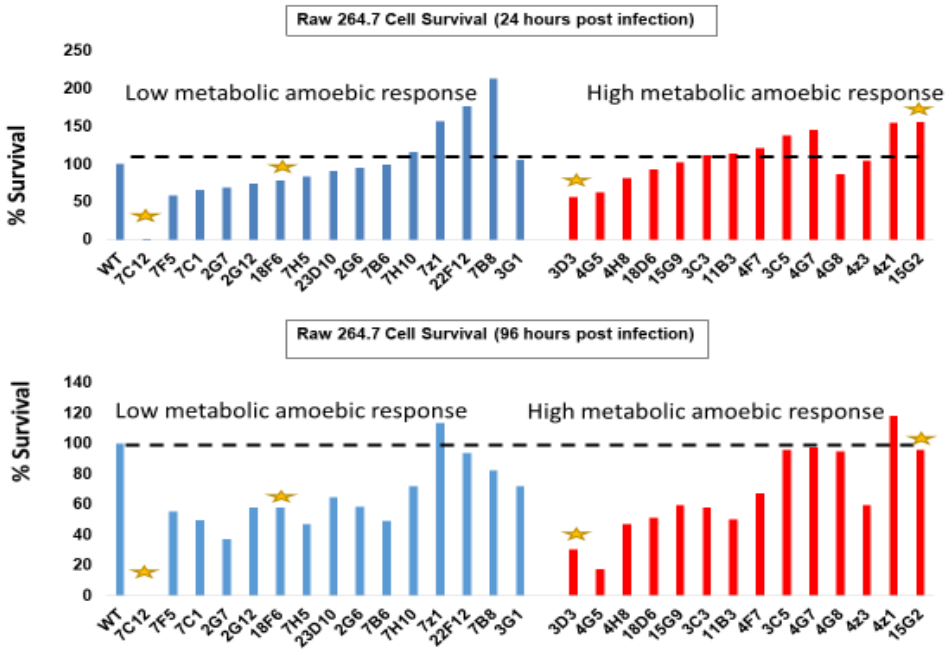

Figure 4a and 4b. Verification of MAP mutants using Raw 264.7 macrophages

To confirm the validity of the amoeba metabolism screen and verify MAP clones are mutants in Raw 264.7 macrophages, macrophage uptake (not shown), 24 hour survival (a), and 96 hour survival (b) of wild-type MAP and each mutant clone was assessed by a 2 hour infection followed by amikacin treatment to eliminate extracellular bacteria. Cells were lysed and intracellular bacteria were quantified at time 24- (a) or 96-hours (b) post-infection. Survival assay values (a and b) are normalized to the original amount of bacteria ingested in the 2 hour uptake assay for each mutant strain. Data represent the mean ± SD of 3 independent experiments each completed in duplicate. Mutants 3D3, 18D6, 15G2, and 7C12 had a reduction in growth and these mutants were chosen for further investigation.

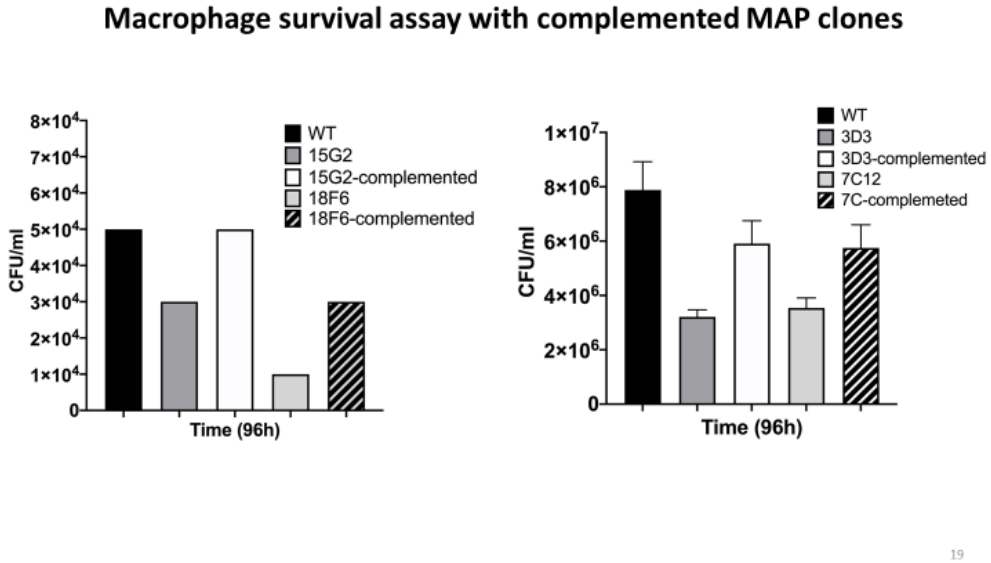

Figure. 5a and 5b 96 hour survival of MAP within Raw 264.7 cells. The mutants, 3D3, 7C12, 15G2, and 18F6 and their complemented mutants containing a complete copy of the gene were infected for 96 hours and the plates were counted after 4 weeks. Cells were infected with bacteria at a MOI of 10, and the number of intracellular bacteria was determined at 4 h after infection as described in Materials and Methods. Values are the means of three experiments standard errors of the mean.

Summary of MAP Tn mutant screen

Mutant	Amoeba metabolism	Gene Name	Domains
23D10	Low	MAP_1423	Synaptic vesicle glycoprotein 2
18F6	**Low**	**MAP_2291**	**Truncated hemoglobins(TB)**
7B8	Low	MAP_1591	Methylmuconolactone or PPOX class
7H5	Low	MAP_2324c	YdfJ Uncharacterized membrane protein/ MmpL
7B6	Low	MAP_1592	Methylmuconolactone or PPOX class
7C1	Low	MAP_3947	Uncharacterized protein/MMPL
7C12	**Low**	**MAP_0949**	**EAL domain/Diguanylate cyclase**
3G1	Low	MAP_2973	Penicillin binding protein
2G7	Low	MAP_0122	PE family
2G6	Low	MAP_2127	Flavin-utilizing monoxygenases
2G12	Low	MAP_1133	Methionyl-tRNA formyltransferase
7B10	Low	MAP_1221	DNA-binding response regulator [Signal transduction mechanisms, Transcription regulator]
2G10	Low	MAP_2363c	Acyl-CoA dehydrogenase related to the alkylation response protein AidB ,Lipid transport
7€1	Low	MAP_1024	Cystathionine beta-synthase
22F12	Low	MAP_1842c	tRNA A58 N-methylase Trm61 [Translation, ribosomal structure and biogenesis]
7F5	Low	MAP_2843c	Hypothetical protein/7TM diverse intracellular signaling

18D6	High	MAP_0338c	Transposase domain
4G8	High	MAP_3717c	Prolyl oligopeptidase family alpha/beta hydrolase fold Serine aminopeptidase
4€3	High	MAP_0294c	Biotin carboxylase/ pyruvate carboxylase
15G9	High	MAP_800c	Helicase
15G2	**High**	**Map_3634**	**IgD-like repeat Mycobacterial L transpeptidases**
14C8	High	MAP_1076	Uncharacterized protein
11B3	High	MAP_2535	Proteasomes endopeptidase
11D3	High	MAP_1642	Metallo-hydrolase-like
7H10	High	MAP_4350c	50s ribosomal protein L34
4G7	High	MAP_1450c	Flavoprotein CzcO
4G5	High	MAP_3832c	HSP70 glutamate dehydrogenase
4H8	High	MAP_1301	ChaA Ca 2/H antiporter
14E3	High	MAP_1320c	Lipid-transfer protein
4H6	High	MAP_3761c	Sulfolipid-1 addressing protein/ SAP
3C3	High	MAP_2228	Pimeloyl-CoA dehydrogenase
3C5	High	MAP_0824	Ferritin Like superfamily
3D3	**High**	**MAP_3893c**	**Serine/Threonine kinase PKnG**
4F7	High	MAP_0847	Domain of unknown Function
11D2	High	MAP_1824c	Transposase and inactivated derivatives, IS30 family [Mobilome: prophages, transposons]

Figure 2.1 Summary of results of the MAP mutant library screen, macrophage validation assay, and mutant sequence

The majority of the mutants caused a change in amoeba metabolism that was similar to that caused by wild type infection. We have found 45 mutants the caused a change in the amoeba metabolism that was 2.5 fold higher than wild type. The mutants with the lowest metabolism were 3.89 times lower than wild-type. Those with the highest metabolism had metabolism 3.5 times that of wild type MAP infection.

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Chapter 3

Pathogenic aspect of *Mycobacterium avium* subspecies *paratuberculosis* dissemination and intracellular survival is revealed through Johne's Disease mimicry with cell culture passage system

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Abstract

Mycobacterium avium subspecies *paratuberulosis* (MAP) is the causative agent of Johne's disease which results in incurable, chronic, and fatal diarrhea in ruminant species and hundreds of millions of dollars in losses for the agricultural industry. Natural infection usually begins with MAP uptake by the epithelium of the small intestine followed by ingestion by tissue macrophages and dissemination throughout the body via the lymphatic or blood system. To gain insights into the adaptation of MAP within phagocytic cells, we employed a mass spectrometric-based quantitative proteomics approach. A cell culture passage model that mimics the interaction of MAP within intestinal epithelium followed by uptake by macrophages and the later release of the pathogen to infect the intestinal epithelium, was previously developed. Using this system, we passed MAP infection of epithelial cells for both 24 hours and 10 days prior to RAW 264.7 macrophage infection (Indirect Infection, II). Uninfected cells and MAP infection without passaging bacteria via epithelial cells (Direct Infection, DI) were used as controls. After 24 hours, cells were mechanically lysed. Trypsin-digested samples were separated by two-dimensional liquid chromatography and analyzed by electrospray ionization tandem Mass Spectrometry. Approximately 2,700 proteins were identified in uninfected, DI and II infection groups. Protein subset comparisons showing more than two-fold expression identified 85, 33, and 39 proteins in DI and II infections with 24h passaged MAP and II with 10 days passaged bacteria, respectively. Heat map analysis for gene enrichment revealed the predominant functional groups in the DI group were related to cytokine signaling, positive regulation of defense responses, and cell activation involved in the immune

response and adaptive immune system. These responses were absent in the 24 hour and II 10 days groups of the macrophage infection. We observed cellular pathway enrichment for cell cycle, healing, cell surface integrins and SNARE trafficking signaling in these groups. We explored the importance of integrins and hypothesize that macrophages infected with passaged II MAP will initiate stronger endothelial cell binding leading to bacterial spread and dissemination. Identification of specific macrophage proteome changes during MAP infection significantly expands our understanding of phagocytic cell signaling pathways, reflecting immune responses that may allow extended survival within host cells.

Introduction

Johne's disease (JD), a severe, chronic, endemic wasting disease resulting in emaciation of ruminant animals worldwide is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Herds afflicted with JD have reduced milk output, increased mortality, and high cow-replacement costs (Carter) (Kovich et al.). The annual costs associated with MAP range from 250 million to 1.5 billion dollars in the US alone (Barratt et al.). MAP control and prevention are possible through strict herd management practices, early detection, and culling of infected animals. Importantly, the link between MAP infection and human Crohn's disease has been investigated with inconclusive results. Human lesions and case presentation are similar between JD in ruminants and Crohn's disease (Waddell et al 2015).

Johne's disease progression is slow, clinical signs of intermittent diarrhea and weight loss doesn't occur until 2-5 years post infection. Some of the factors which allow this bacterium to grow unabated within host macrophages, as well as to evade elimination, have been characterized. Other factors contributing to the long incubation period and deficient immune response remain unknown. Neonatal and young ruminants are exposed to MAP while nursing on infected dams and through fecal contaminated milk and water sources. In-utero infection can also occur. The organism arrives at the distal small intestine where it enters the lamina propria through Microfold cells and enterocytes (Pott et al 2009) (Bermudez et al 2010). The invading bacteria is then taken up by local tissue macrophage and dendritic cells.

Macrophage infection is central to JD pathogenesis as the phagocytic cell supplies the nutrients for the bacteria to replicate and thrive. Although the silent and subclinical

stages of infection are not fully understood, at some point, the disease progresses, and affected animals begin to show clinical signs of profuse watery diarrhea and loss of body condition. The silent phase of the infection, in which the host does not show signs of disease, is followed by an inflammatory phase in which there is shedding of the bacterium throughout the intestinal tract.

We published a novel *in vitro* cell passage model based on our current understanding of MAP pathogenesis (Everman et al.). This culture passage model mimics natural infection, invasion of the intestinal epithelium followed by infection of the host phagocyte finally returning to the intestinal epithelium. In this sequential cell passage model, the initial infection is not associated with an inflammatory response. This *in vitro* model system demonstrated identical bacterial gene expression compared to the intestinal mucosa of cattle (Everman et al.). It was also found that bacteria passed through epithelial cells and macrophages elicit a pro-inflammatory immune response. The diverse phenotypes of MAP reflect the multiple disease stages during the course of infection. These varying bacterial phenotypes could play an important role in addressing inconsistent diagnostic results and vaccination efficacy (McBride and Walker).

Past work has demonstrated that the change in phenotype is associated with components of the bacterial cell walls (Everman et al.). Host molecular profiles are altered during bacterial phenotypic changes. To gain insights into host metabolic and regulatory defense cellular pathways associated with an intracellular phenotype and divulge MAP adaptation within phagocytic cells, we employed a mass spectrometric-based quantitative proteomics approach. We found that macrophages infected with

MAP that has been first passed through bovine epithelial cells (Intracellular Infection (II)) develop significantly lower inflammatory response compared to direct phagocytic cell infection. We observed cellular pathway enrichment for cell cycle, healing, cell surface integrins and SNARE trafficking signaling. Conversely, in the non-passaged model (Direct Infection (DI) where macrophages are infected directly, we saw significant pro-inflammatory responses and upregulation of pathways involved in microbial killing, immunity activation, and cytokine secretion. Analysis of the proteins involved in the different infection stages led to confirmation assays which support our overall findings.

Methods and Materials

Bacterial preparation. *Mycobacterium avium* subspecies *paratuberculosis* strain K10 (ATCC BAA-968) was cultured at 37°C on 7H10 agar supplemented with casein hydrolysate (1g/L; BD), 10% (v/v) oleic acid, albumin, dextrose, and catalase (OADC; Hardy Diagnostics; Santa Maria, CA), and ferric mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) for 4 -5weeks. Prior to experiments, a bacterial suspension was made in PBS, passed 15 times through a 1.5 inch, 22-gauge needle to disperse clumps, and allowed to settle for 10 minutes. The top 3/4 of the inoculum was used as a single-cell suspension for experiments as previously described (Patel, Danelishvili et al. 2006).

Mammalian cell culture. Madin-Darby bovine kidney (MDBK) epithelial cells (CCL-22) and RAW 264.7 macrophage cultures (TIB-71) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Both cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products; West Sacramento, CA) at 37°C in 5% CO_2. Proliferating Bovine Aortic Endothelial Cells (BAOEC) were obtained from Cell Application INC (San Diego, CA). BAOEC were cultured in Bovine Endothelial Cell Growth Medium at 37°C in 5% CO_2. All cell lines were maintained according their respective manufacturer's protocols.

Invasion and survival of MAP in Raw 264.7 cells during direct and intracellular infection. MDBK cells were seated to a 75% confluency in T-75 flasks prior to MAP infection. Bacteria were prepared as previously described and cells were infected for 24 hours. Cells were then lysed in 0.1% Triton x-100 (Sigma-Aldrich St. Louis, MO)

and the resulting bacteria used in Intracellular Infection (II) assays. Monolayers of Raw 264.7 were seeded in 24 well plates in DMEM overnight then exposed to DI and II bacteria. Plates were synchronized by centrifugation at 250 RPM for 5 minutes then incubated at 37°C for two hours. The supernatant was then removed and the wells washed three times with Hank's buffered salt solution (HBSS). DMEM media with amikacin (200 g/ml) was added for 2 hours at 37°C to kill extracellular bacteria. Afterward, monolayers were lysed with 500uL of 0.1% Triton X-100 in HBSS for 15 min. 100 uL was taken from each lysate, serially diluted, and plated onto 7H10 agar enriched with 1 gram casein hydrolysate, 100 mL of oleic acid, albumin, dextrose, and catalase (OADC) and 2 mL ferric Mycobactin J to quantify colony forming units (CFUs). Monolayers and plating were repeated at 24 and 96 hours for MAP and mutant survival assays.

***In vitro* cell culture passage model preparation for quantitative proteomics.** *In vitro* cell infection was performed according to Figure 1. MDBK cells were grown to 75%-80% confluence then infected using MAP prepared as described above at 50-100:1 for 4-6 hours. After incubation, the media was removed, cells were washed with phosphate buffered saline (PBS) twice, and fresh media was added. After 24 hours, modified differential centrifugation was used to isolate bacteria from host cells (Lodish et al.). Cells were lysed with 0.1% Triton X-100 for 20 minutes. Lysates were collected and centrifuged for 15 minutes at 500 × RPM for 5 minutes at 4°C. The pellet was resuspended in PBS then centrifuged for 30 minutes at 3500 × RPM at 4°C. This bacterial pellet was again resuspended in PBS and centrifuged at 500 × RPM at 4°C to pellet any residual cell debris for 5 minutes. Finally, the MAP-

containing supernatant was collected and centrifuged at 7,000 × RPM at 4°C to pellet intracellular bacteria for 10 minutes. The bacteria were resuspended in DMEM medium described earlier. This inoculum was added to T-25 flasks of Raw 264.7 cells for 4 hours. The wells were washed with PBS twice followed by the addition of fresh media. For direct infections (MAP grown on agar plates), the inoculums and infection of Raw 264.7 cells were done as described for II cells. After 24 hours, cells were mechanically lysed with 3% sodium dodecyl sulfate (SDS Millipore, Sigma, St. Louis, Mo) supplemented with the protease inhibitor cocktail (ThermoFisher Scientific). Samples were submitted to the Oregon State University Department of Chemistry Mass Spectrometry Center. Trypsin-digested samples were separated by two-dimensional liquid chromatography and analyzed by electrospray ionization tandem Mass Spectrometry.

Protein analysis

After examining the proteins in a variety of methods we used the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System to order proteins (and their genes). Proteins are grouped by evolutionarily related proteins, molecular function, biological process, or by pathway. The group of related proteins called integrins were found to be differentially expressed between the direct, II 24 hours, and II 10 day subsets of infected Raw 264.7 cells.

Binding Assay. Cell cultivation and infection schedules were completed as described for the *in vitro* cell culture passage model for creation of II Raw 264.7 cells with 10 MOI. After 24 hours of infection, a 1:1000 dilution DAPI, or 4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to adenine–thymine-rich regions

in DNA, was added to the DI, II , and uninfected Raw 264.7 macrophage cells. After thirty minutes, the cells were washed three times with PBS. Cells were then detached from the 24 well plates using 30% uM cold EDTA. The cells were quantified and adjusted to 1 x 10^5 cells/mL and resuspended in 300 uL of Bovine Aortic Endothelial cell media. The cells were then added to a confluent population of Bovine Aortic endothelial cells seeded at 2x10^5 cells/mL for two hours. After two hours, the mixed population of endothelial cells and macrophages were washed three times with PBS. Fluorescent readings for uninfected, DI, and II Raw 264.7 cells were measured using the TECAN infinite 200 pro-trading AG, Switzerland

Endothelial cell migration transwell assay. The experimental protocol for infecting cells were described under binding assay. Bovine aortic endothelial cells were seeded on a 20 um transwell filter. The initial transwell membrane resistance was 80 uOm. The cell membrane reached confluency at day 5 with a resistance reading of 280 uOM. No leaks were seen when trypan blue was added to wells. MAP infections were set up as the following experimental groups: an uninfected control, DI, and II. Raw 264.7 cells were seeded on a 20 um filter transwell for 2 hours and 24 hours. The cells were stained with Giemsa and counted using a brightfield microscope.

Statistical analysis and data interpretation. Results are reported as the mean of at least 2 independent experiments ± standard error. For binding assays, statistical comparisons between experimental groups and control groups were determined using the Student's *t* test with $p<0.05$ denoting statistical significance. Survival curve data was analyzed using Kaplan Meier Survival Analysis. GraphPad Prism version 6.0

software was used for the construction of graphs, data interpretation, and all statistical analysis

Results

Macrophage model description

To readily understand the progression of Johne's pathogenesis, our lab developed an *in vitro* cell culture passage model that mimics the interaction between the bacterium and the host intestine over the course of infection (Everman et al.). Our goal is to understand how MAP manipulates the host mechanisms after crossing the intestinal epithelial barriers at a molecular level. Figure 1 schematically represents the intestinal experimental design showing bacteria entering the epithelial cells (MDBK cells) as a model of bacterial entry into host enterocytes. Bacteria then leaves the epithelium to be ingested by macrophages where they replicated and disseminate throughout the body. It is unknown if MAP enters enterocytes then immediately transverses to the lamina propria to enter macrophages, or, remains within this stage of infection for an extended time frame. To explore both possibilities, infected epithelial cells were analyzed for both 24 hours and 10 days. After 10 days of infection, we observed that infected cells remained visually intact and appeared healthy. Importantly, the bacterial cells remained within the cells and did not exit to the supernatant. After ten days, we lysed the MDBK cells to recover the bacterium for the next stage of infection.

Passage of MAP increases uptake and survival within the macrophage

It was established that the passage of bacteria through the epithelium results in bacterial phenotypic changes (Everman et al.). In Figure 2, we investigated if this phenotypic change impacts the bacteria's ability to be phagocytized and survive within the host. For indirect infections (II), MDBK cells were infected with MAP for 24 hours. The MDBK cells were lysed to recover MAP which was then used to infect Raw cells. Macrophage infection timepoints were 4 hours, 72 hours, and 120 hours. These time points were chosen to mimic early and late stages of infection. After being plated and allowed to grow for 4 weeks, we found differences in survival and uptake between direct and indirect infections. Percent uptake was increased at 4 hours during II. Over the course of the infection, there was a slight increase in bacteria survival of passaged bacteria compared to non-passaged bacteria. Our results suggest passage through the epithelium alters the bacteria, allowing for better recognition and uptake and survival within macrophages.

Macrophage protein profile changes during *in vitro* passage model

To more precisely determine how bacterial phenotypic changes during infection may alter the host response, we used the *in vitro* cell culture passage model for quantitative proteomics. Plate grown bacteria (Direct Infection (DI) or non-passaged) and 24 hours or 10 days epithelial cell passaged bacteria (Indirect Infection (II) or passaged) were used to infect Raw 264.7 cells for 24 hours. Uninfected macrophages were passaged with HBSS to serve as controls. Cells were lysed to extract proteins synthesized during infection for proteomic analysis. Approximately 2,900 total

proteins were expressed after 24 hours in the DI, II, and uninfected groups (Figure 3.1). 252 proteins from the DI were upregulated; 115 were found to be statistically significant (Figure 3.2). 227 proteins expressed during the 24-hour II were upregulated of which 77 were statistically significant (Figure 3.2). 237 proteins were upregulated in the indirect 10 day infection but only 78 were statistically significant (Figure 3.2). Figure 3.3 is a Venn diagram of proteins that were significantly down regulated within the three infection groups. The heatmap of gene ontology (GO) categorizes the proteins into KEGG pathways. These pathways are collections of pathway maps representing molecular interactions, reactions, and relationship networks. We found that these systems were different between the direct and indirect 24 hours and ten-day groups. Gene ontology for DI proteins included cytokine-mediated signaling, cytokine production, responses to interferon-gamma, cellular responses to lipopolysaccharide, regulation of Nf-kappa B kinase signaling, regulation of the inflammatory response, cytosolic DNA-sensing pathway, and positive regulation of defense responses (Figure 4.1). The II 24 hours and II 10 days pathways included NABA extracellular matrix regulation, maintenance of location, and hyaluronan metabolic process (Figure 4.1). Comparing the II 24 hours and II 10 days proteins, we found some overlap of important pathways including those associated with ECM, exocytosis, insulin-like growth factors, wound healing, and interleukin 4 and 13 signaling (Table 1 and Figure 4.2). These findings confirm the anti-inflammatory phenotype of host immune cells. However, the II 24 hour profile highlighted pathways for nitric oxide processing and regulation of oxidative stress which could indicate that this particular infection type is more inflammatory than the

II 10 days (Figure 4.2). These results implicate that extended dwelling in the enterocyte or epithelial cell has a positive impact on the ability of MAP to remain undetected by the host immune system. Figure 4.3 is the GO of the proteins which are downregulated in all three groups. Proteins associated with apoptosis were found to downregulated in all groups. As expected, cytokine signaling was downregulated in both of the indirect infection groups while proteins associated with the TCA cycle were downregulated in the direct infection groups.

Integrin upregulation

Table 1 shows the integrin proteins of interest. Itga5, Fn1, CCL4 proteins were upregulated in all groups. Adam8 was upregulated in the DI protein group but severely downregulated in the II 24 and II 10 days. Protein comp/fibulin1, CD36, and Itgam were upregulated in the II 24 hour, II 10 days. Interestingly, Itgam was severely downregulated in the DI infection.

Binding assay and Endothelial migration transwell assay

Figure 6.1 shows that after 1 hour of endothelial cell monolayer exposure, the II macrophages attach more firmly after three washes. The level of fluorescence was greater in the II exposure of macrophage to BAOEC than in the DI infection. This indicates that the upregulation of these integrins are associated with a stronger attachment to the endothelium and possible movement to distance location throughout the body. To examine if the attachment led to actual migration through a membrane, we utilized a polarized membrane and quantified the resulting number of cells. Figure 6.2 shows that the number of macrophages crossing the endothelium was much higher in the DI group.

Discussion

Tissue macrophages and dendritic cells play a crucial role in the establishment, maintenance, and progression from silent infection to advanced clinical JD (Weiss and Souza). The inability of immune cells to eliminate the pathogen from the host is central to the long incubation period before the animal progresses to clinical and advanced clinical disease stages. To understand the molecular mechanism underlying MAP's prolonged survival within the ruminant host requires the ability to study this pathology in a controlled research environment. A model of JD which accurately depicts the precise host manipulations of the immune system is not available. *In vivo* methods have been developed to study MAP pathogenesis (Begg and Whittington). Breed, age of inoculation, species, MAP strain used, route and dosage of infection all influence experimental outcomes (Begg and Whittington). Replication of experiments is uncommon for *in vivo* ruminant studies and animals per group are typically few in number. Ideally natural hosts (cattle, goats, and sheep) should be used to study JD but disease progression over a 2-5 year span incurs tremendous expense in facilities, feed, and husbandry. Alternatively, researchers have turned to the use of rodents and lagomorphs to study JD evolution with little success. All of the above combined complicates interpreting and concluding *in vivo* data.

In vitro modeling dominates the majority of JD host pathogen interaction studies. Recently, researchers are attempting to develop more complex models of Johne's disease (Marfell et al.) (Casey et al.) (McLoughlin et al.). The multicell model used in this study provides a different approach to *in vitro* MAP studies. It was

hypothesized that MAP uses the epithelium to somehow enhance its ability to survive after crossing into the lamina propria and being phagocytized by macrophages or dendritic cells. This strategy nullifies macrophage defenses against the pathogenic invader. Therefore, macrophages exist as a nidus of infection until later disease stages when MAP leaves the host to enter the environment. The 24 hour infection of the macrophage in our model represents an early time point for synthesized proteins. In the host, MAP can persist for extended amounts of time and require the intracellular environment for efficient growth, replication and evasion of the immune system. Our goal was to discover which proteins are synthesized or blocked in order to curate or establish an environment suitable for the pathogen. We collected proteins from lysed macrophages infected by bacteria directly from the agar plate and from those infected by bacteria which had been passaged. The *in vitro* passage models first described in our lab represent a novel technique in which intricate host-microbe interactions during various stages of infection may be analyzed in a simplified manner.

Tissue macrophages are extremely dynamic as innate immune cells, playing major roles in normal physiology, pathology, and healing (Wynn et al 2013). In the pro-inflammatory stage, macrophages efficiently defend against microbial agents (Weiss and Souza). They secrete a variety of inflammatory mediators including nitric oxide, a variety of cytokines and chemokines, and vasoactive proteins. The initial contact between infectious agents and macrophages or dendritic cells typically lead to opsonization, oxidative burst, changes in intracellular trafficking and phago-lysosomal acidification (Hostetter et al 2005). Cytokines produced by these macrophages recruit a large number of immune cells to the site of the infection

including dendritic cells, B cells, T cells, and additional phagocytes ((Tailleux et al.).The majority of viral, fungal and bacterial organisms are eliminated successfully through these mechanisms. The dynamic nature of macrophages are exploited by pathogens to aid in their own survival and propagation. The “phenotypic diversity” leads to pro-inflammatory or anti-inflammatory conditions. It is well established that macrophage activation is essential to MAP control (Atri et al.). Like most intercellular infections, this is dependent on a T helper lymphocyte type one (Th1) response.

All pathogenic mycobacteria have achieved success as intracellular pathogens through manipulating host macrophages in order to reside and replicate within them (Cook et al.). The pathogens are not destroyed by innate defense mechanisms. Macrophage activation by interferon-gamma (INF-γ) is required to destroy MAP (Thirunavukkarasu et al.) Mice deficient in tumor necrosis factor or tumor necrosis factor-receptor I (TNF-RI) challenged by other mycobacterial species like *Mycobacterium tuberculosis* (*Mtb*) are extremely susceptible to low-dose aerosol infection (Flynn et al., 1995). Similarly, mice deficient in interleukin-1a and IL-1b or IL-1RI are extremely susceptible to low-dose aerosol *Mtb* infection (Cooper et al.). SNARES (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) were especially upregulated in that study. These are endosomal and lysosomal proteins responsible for membrane fusion and movement of organelles throughout the cells. SNAREs are vital in viral infection, cell fertilization, intracellular transport, and neurotransmitter release (Garg and Joshi). Many SNARES and mobility proteins were upregulated in the II group of our study. One identified SNARE trafficking protein,

Rab32, was upregulated during II. Rab32 is a member of a large family of small GTPase that control membrane identity, vesicle formation, motility, and fusion (Stenmark). Rab32 is associated with vesicle remodeling and maturation. Hu et al 2019 showed that the host cell Rab32 plays an important role in mediating antimicrobial activity by promoting phagosome maturation at an early phase of infection with *B*. *pseudomallei* (Hu et al.). Upregulation of this protein during infection leads to elimination of the pathogen by lysosomal degradation while blocking this protein should allow growth and persistence of the pathogen. It is unknown what purpose this protein served in the initial and later stages of MAP infection.

Our proteomic results indicate that MAP passage through epithelial cells upregulates integrin expression. The DI cohort displayed integrin downregulation. The effect of MAP on immune cell integrin expression has not been explored in recent literature. Given the prominent role of these integrins in migration, adhesion to vascular endothelium, and interactions with T cells, we plan to investigate the influence of MAP infection on integrin expression. Our results to date demonstrate that macrophage exposure to MAP results in an increase in β integrin subunit expression in epithelium-passaged cells with impaired macrophage adherence to endothelial cells in directly infected macrophages. Lymphatic endothelial cells activate cell surface molecules and ligands during various disease states. These integrins play a crucial role in immune responses and initiation (Hampton and Chtanova).

The integrin ITGAM/ITGB2 was 4x and 3x upregulated in the II 24 hours and II 10days cohorts, respectively. The same integrin was markedly downregulated in the DI infection. This integrin has important purposes of adhesive interactions for monocytes and macrophages. The β2 subunit is responsible for immune cell attachment to endothelial cells and migration within the extracellular matrix. When these integrins are blocked using antibodies to CD18, CD11a, CD11b and VLA-4, phagocytic adhesion to and transmigration through endothelial cells is inhibited.

The Itga5 protein integrin is an extracellular matrix receptor that acts as an adhesive receptor for extracellular matrix proteins, including fibrin, adipocytes, laminin and collagen (Zou et al.). ITGA5 forms the link between the extracellular matrix and intracellular signal transduction, and also participates in a variety of important physiological processes (Morandi et al.). It is also associated with tumor occurrence, development, invasion and metastasis (Zhang). ITGA5 as a cell surface receptor mediates *Mycobacterium* entry into macrophages challenged with high MOI (Park et al.).

The final extracellular matrix molecule of interest is CD36 (Park et al.). In our study, CD36, a member of scavenger receptor B family, is a transmembrane glycoprotein receptor expressed in a variety of cells including adipocytes, endothelium, epithelium, myocytes, platelets cells, monocytes, and macrophages (Silverstein and Febbraio). Recent studies have revealed that macrophage CD36 functions as a signaling molecule, transmitting signals via specific Src and MAP kinases Lyn and JNK1 and JNK2 upon binding to ligands or oxidized low density lipoprotein (Guy et al.). These signals are required for oxLDL internalization and

foam cell formation (Silverstein and Febbraio). CD36 is involved in several physiological and pathological processes including immunity, lipid absorption, storage and metabolism, inflammation, and cardiovascular and Alzheimer's disease.

Several proteins important for the movement of cells throughout the extracellular matrix were upregulated in the II group. Results from the binding assay and endothelial cell transwell migration assay show that passage of MAP through epithelial cells alters bacterial manipulation of host cells. This was confirmed by two separate biological tests, the endothelial binding assay and the polarized membrane migration assay. These assays test the ability of DI or II macrophages to attach then migrate through a polarized membrane of endothelium cells. Our hypothesis is that the upregulation of integrins in the II macrophages allow for more effective attachment and transport through the endothelium than the DI macrophages. These results suggest that the upregulation of the integrins in the II cohort might change the ability of the macrophage to attach to the endothelial cells, allowing them to disseminate throughout the host.

The ultimate goal of this study is characterizing how innate immune cells respond to changing bacterial phenotypes. These data provide a new perspective of Johne's disease pathogenesis. Proteomic studies provide a refreshing source for interrogating complex disease processes. Massive data banks can only be useful when navigated using current literature and a thorough understanding of the subject matter. It is important to pair proteomic findings with biological assays in order to validate proteomic findings. In this study we used both bioinformatics and branch top assays to investigate the host pathogen interaction.

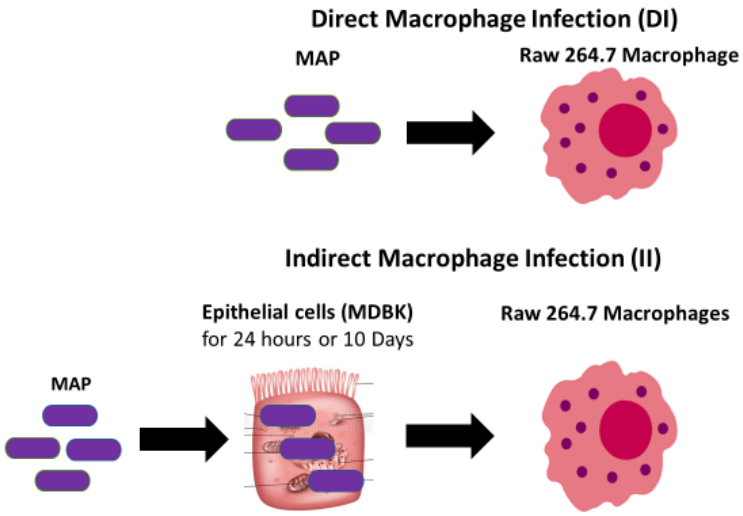

Figure 1 : *In vitro* cell culture passage model. A schematic of the cell culture passages used to mimic the path MAP takes during infection. The timepoints for the indirect infection include 24 hours and 10 days. The samples collected for analysis of host-microbe interactions during cell culture passage are shown below.

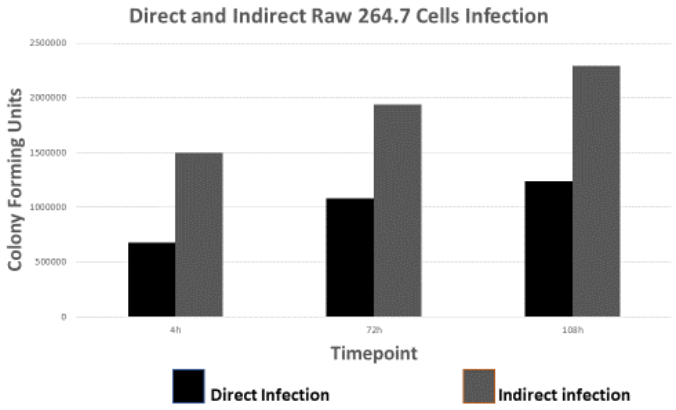

Figure 2 *In vitro* survival and uptake assay. Raw 264.7 cells were seeded and infected with MAP over the course of five days. Time points include 4 hours, 72 hours, and 120 hours, when cells were lysed. Released intracellular bacteria were plated and colony forming units were quantified after 4 weeks for all time points.

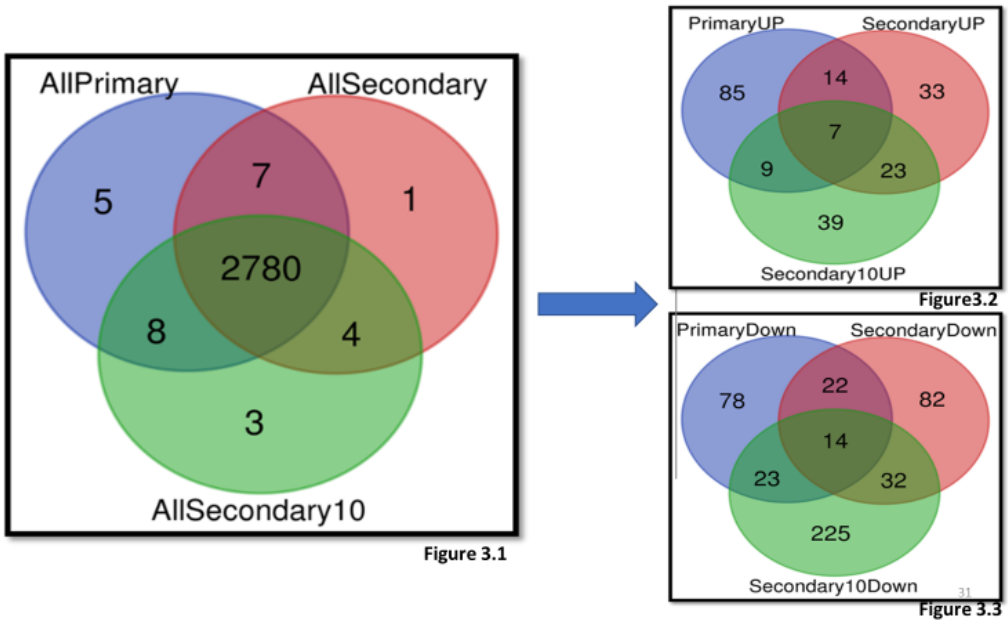

Figure 3.1. Venn Diagrams of all groups. Approximately 2,900 proteins were identified in uninfected, direct, and indirect infection groups. **Figure 3.2** represents proteins which are statistically significant and upregulated. **Figure 3.3** demonstrated downregulated proteins in all groups. The Venn Diagrams were made using software for the http://bioinformatics.psb.ugent.be/webtools/Venn/ website.

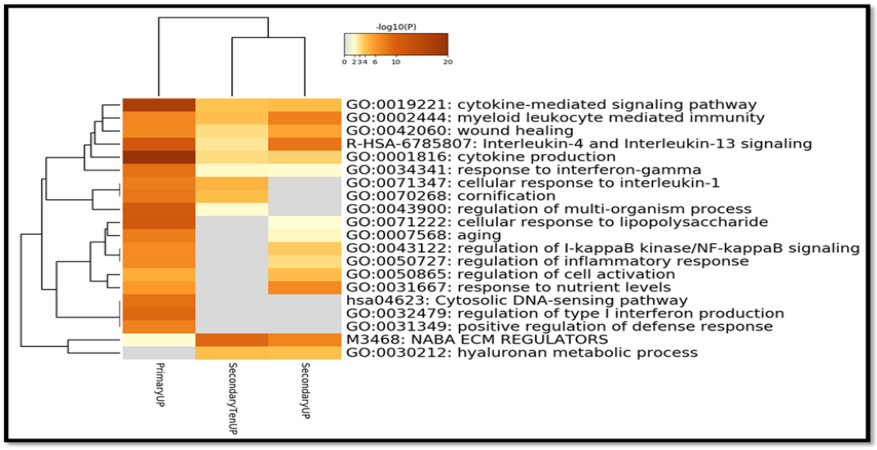

Figure 4.1

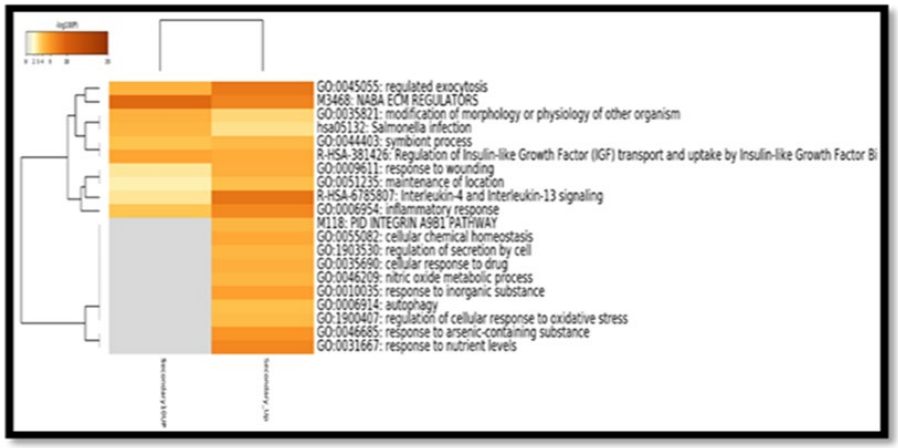

Figure 4.2

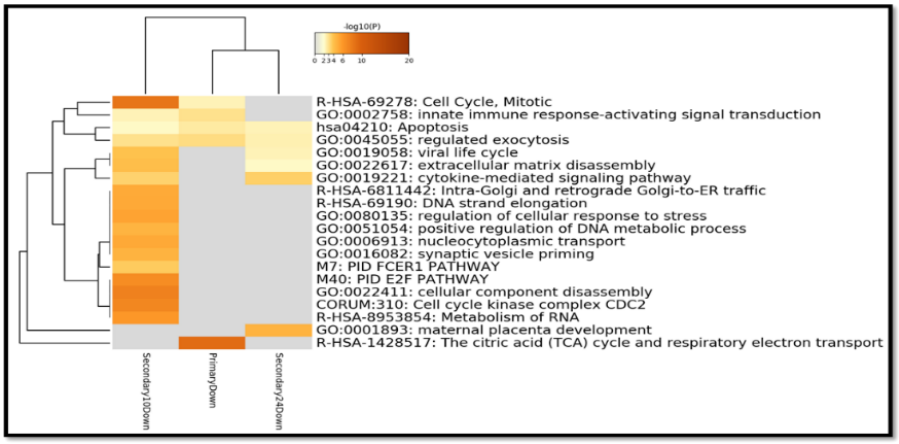

Figure 4.3

Figure 4 Gene Ontology and Pathway enrichment analysis. The pathway and process enrichment analysis was performed using Metascape (http://metascape.org) for host proteins uniquely synthesized in MAP infected macrophages with statistically significant changes. The groups include the direct infection group, indirect infection 24 hour and the indirect infection group 10 days. **Figure 4.1** Heatmap of proteins that were upregulated. **Figure 4.2** Geno ontology and pathway enrichment for pathways found between the II ten days and the II 24 hours groups alone. **Figure 4.3** Gene ontology heatmap of downregulated proteins in all groups.

Infection group	Pathways upregulated
Direct infection	Microbial killing, Immune system activation, Cytokine-mediated signaling, Cytokine production, Response to interferon-gamma, Cellular response to lipopolysaccharide, Regulation of I-kappa B kinase signaling, Reactive oxygen species metabolism, DNA-sensing pathway, defense response
Indirect Infection 24 hour	Protein secretion, Reactive oxygen species metabolism, ECM regulation, hyaluronan metabolism, exocytosis regulation, symbiont process, Regulation of Insulin-Like Growth Factor
Indirect Infection 10 Day	Wound healing, Maintenance of location, ECM regulation, hyaluronan metabolism, exocytosis regulation, symbiont process, Interleukin-4 and IL 13 signaling, cellular homeostasis, Autophagy, Regulation of Insulin-Like Growth Factor, Regulation of oxidative stress, Nitric oxide metabolism, response to nutrient levels

Table 1 Summary of Pathways Upregulated in the direct, indirect 24 hours, and the indirect 10 days infection groups, as indicated by proteomic analysis.

SNARES, Endosomal, and Lysosomal proteins	Infection group	Role of Protein during infection
Vamp8	II 24	Regulates autophagosome–lysosome fusion
Vamp3	DI and II 24	SNARE involved in vesicular transport from the late endosomes to the trans-Golgi network
Vps18	II 24 and II 10 days	Protein trafficking, formation of early endosomes, late endosomes, and lysosomes
Lamtor1	II 24	Lysosomal motility; activation on the late endosome as well as endosomal biogenesis
Rab32	II 24	Regulators of membrane trafficking pathways in eukaryotic cells Endosome-mediated membrane trafficking
Rab3iL1	II 24	regulates synaptic vesicle exocytosis
Hpse	II 10 days	Endoglycosidase that cleaves heparan sulfate proteoglycans
Glb1,Hexb, Gm2a	II 10 days	Cleaves beta-linked terminal galactosyl residues from gangliosides
Cathepsin D	II 24 and 10 days	Acid protease active in intracellular protein breakdown
Arsb	II 10 days	Lysosomal transport, autophagy, degranulation of neutrophils
CD63	II 10 days	Intracellular vesicular transport processes
SypL1	II 10 days	Regulates exocytosis; small cytoplasmic transport vesicles
Vps37c	DI	Required for the sorting of ubiquitinated transmembrane proteins into internal vesicles of multivesicular bodies
Litaf	DI	Lipopolysaccharide-induced tumor necrosis factor-alpha factor: Plays a role in endosomal protein trafficking and in targeting proteins for lysosomal degradation
SLCL5a3	DI	Lysosome Peptide/histidine transporter
Ar18a	DI	Plays a role in lysosome motility

Table 2 Highlights of proteomic analysis of different infection groups and proposed function of identified proteins. Direct infection (DI), indirect infection 24 hours (II 24 hours), and indirect infection 10 days (II 10 days)

Integrin or binding protein	**Direct (DI) or Indirect infection (II)**	**Role of Protein during infection**
Itga5	All groups	Composed of an alpha subunit and a beta subunit that function in cell surface adhesion and signaling
Adam8	Upregulated in DI and severe downregulated in II 24 and II 10 days	A disintegrin and metalloprotease domain implicated in a variety of biological processes involving cell-cell and cell-matrix interactions
Comp/Fibulin1	II 24 and II 10 days	ECM protein that stabilizes collagen and other ECM proteins
Fn1	All groups	Cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis
CCL4	All groups	Secreted and has chemokinetic and inflammatory functions
CD36	II 24 and II 10 days	Involved in a variety of adhesive processes
Itgam	II 24 and II 10 days. Severely downregulated in DI infection	Adherence of neutrophils and monocytes to stimulated endothelium, and also in the phagocytosis of complement coated particles

Table 3. Upregulated integrins identified by and documented role in infections

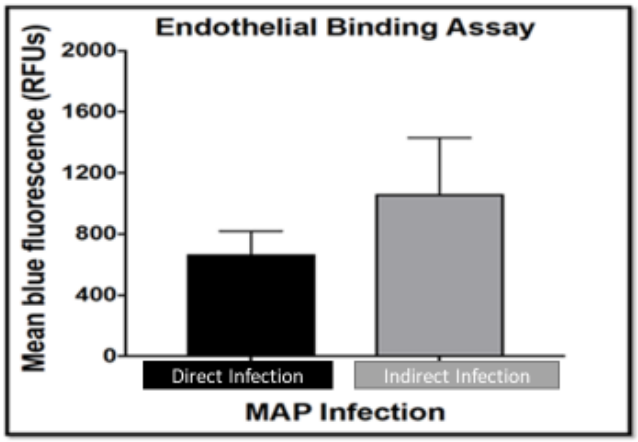

Figure 6.1

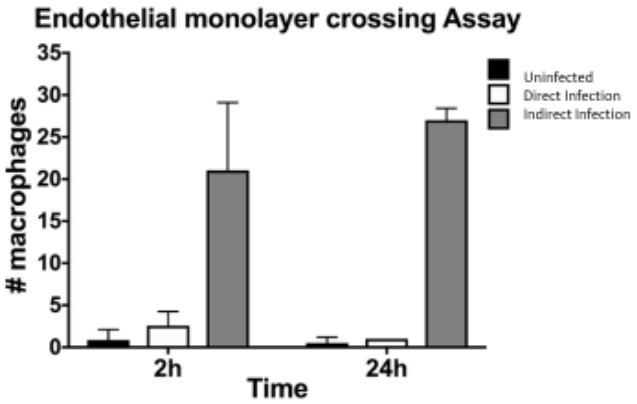

Figure 6.2

Figure 6.1 Endothelial binding assay: Raw 264.7 macrophages were infected with bacteria directly from the plate or with bacteria which has been passed through epithelial cells. These results suggest that the upregulation of the integrins might change the ability of the macrophage to attach to the endothelial cells. **Figure 6.2.** Transwell assay measuring the number of uninfected, direct infected, and indirect infected Raw 264.7 cells which passed through a polarized membrane at 2 hours and 24 hours.

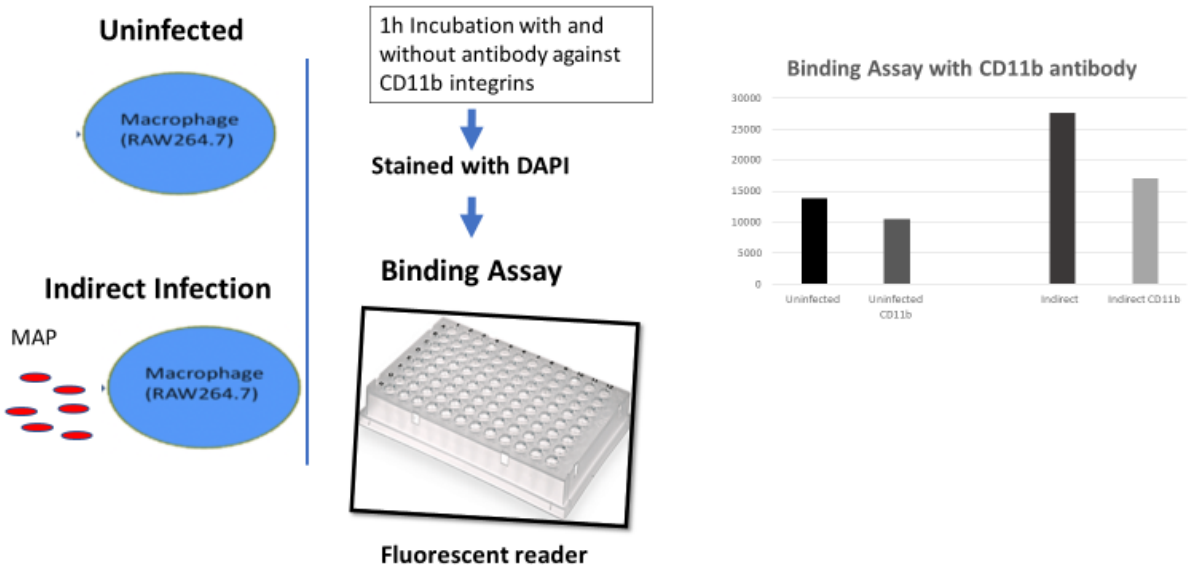

Figure 7. Binding Assay with CD11b antibody. The endothelial binding assay was repeated as described previously with the addition of antibody against CD11b.

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Chapter 4

Conclusion and Discussion

Conclusions

The silent, subclinical, and clinical stages of the Johne's disease makes studying MAP associated pathogenesis very difficult. A complete understanding of this disease requires identification of specific processes, including bacterial virulence factors and various host responses, that influence each disease stage. As MAP changes phenotypes under a variety of environmental and host conditions, we hypothesized that the bacterium alters its phenotype over its long and complicated evolution within the bovine intestinal mucosa.

The interaction between MAP and the ruminant host macrophage is a complex interplay between a host cell with the primary goal of eliminating an intracellular pathogen and a bacterium determined to survive. MAP has developed a collection of functions to evade host-mediated mechanisms and survive within the intracellular phagosome. A number of these bacteria-mediated techniques have been elucidated which include blocking of phagolysosome fusion, blocking macrophage reactions by interfering with nitric oxide formation, interferon gamma signaling and promoting or inhibiting apoptosis as necessary. There are a variety of interactions that are yet to be understood between the host phagocyte and MAP during infection. By using *Acanthamoeba castellanii* as a model phagocyte in Chapter 2, we analyzed the interaction between MAP and the host phagocyte. We focused on the interaction between the bacterium and the metabolic activity of the host phagocyte with the goal of determining some of the bacterial virulence factors.

Our previous studies demonstrated that MAP actively stimulates the metabolic activity of the amoeba host over the course of infection. To determine the bacterial

mechanisms involved in this phenomenon, we identified MAP mutants which demonstrate a hyper-or deficient stimulation of amoeba metabolism. Recognizing that amoeba may not provide a completely identical or relevant host for identifying metabolic MAP mutants, we also used host macrophages to confirm that each identified mutant strains also demonstrated a defect in the ability to infect and/or survive over the course of 96 hours. We then investigated these mutants and their genetic deficits to gain insight into their function to the overall pathogenicity of MAP. MAP genomics have wide applications for identifying virulence determinants, drug targets, attenuation targets for vaccine development, and/or diagnostic antigens. Among other applications, our findings promote the development of novel live-attenuated vaccines (LAV) candidates. The advantages of LAV is that they have been shown to stimulate cell-mediated immunity in mice and ruminant models of JD and are easy to produce and manufacture.

Modeling the intestinal interaction of MAP within the host over the entire continuum of Johne's disease is problematic. Cattle, sheep, and goats are the natural host of MAP infection, yet these ruminant models are costly, necessitate enormous quantities of time, and can generate unreliable results due to individual immune variation. On the other hand, cell culture models are useful, but can oversimplify the complicated range of host-pathogen interactions. Our lab previously developed a method of studying the changing phenotypes of MAP over the course of disease.

Our model is simple and cannot fully represent deep intricacies present in the living multiorgan individual. True intestinal compartments (lining epithelium, mucosa, submucosa, muscle layers, and serosa) as well are the intact immune system

are lacking. Therefore, we cannot conclude that the proteins produced in our system reflect those produced *in vivo*. However, our findings in Chapter 3 strongly incriminate a shift in the immune response from one that is highly inflammatory to eliminate the pathogen, to one that shelters the invader. The limitation of *in vitro* infection models is that while they may be able to derive information about mechanisms at play, they are not representative of the process that is occurring in the natural intact host environment. For instance, how would cytokines generated by other components of the immune response affect this host cell-pathogen interaction? The results generated using this *in vitro* model correlate with inflammatory phenotype transcripts from MAP-infected bovine intestinal tissue biopsies. The next steps to further establish our findings might include proteomic analysis of intestinal biopsies. By illustrating the that proteins found in the indirect infection were also found at higher levels in animals exhibiting a more severe disease state, we would successful validate our model and its ability to identify not only the appropriate phenotype as described in this study, but its ability to be used as a model system for future investigation of Johne's disease mechanisms.

The findings from this proteomics cell culture model will successfully contribute to the tools used to study and understand Johne's disease. This analysis of protein profile composition and components could greatly enhance the field of Johne's research and prevention by promoting more sensitive and specific assays for diagnostic purposes. The knowledge that MAP expresses a different inflammatory phenotype during the various stages of infection, and identification of host markers

like Rab32 or CD 36, provides a toolbox of new biomarkers to be used for JD diagnostics, vaccine development, and intracellular infection research.

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