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Identifying genes in Mycobacterium avium subspecies paratuberculosis that are essential for survival in dairy calves

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IDENTIFYING GENES IN *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* THAT ARE ESSENTIAL FOR SURVIVAL IN DAIRY CALVES

by

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic wasting disease common in dairy cattle. Currently, there are no effective vaccines to prevent infection and fecal bacterial shedding. To determine potential targets for a live-attenuated vaccine strain 12 Holstein-Friesian dairy calves were inoculated with 5 x 10^{10} CFUs on two consecutive days with a mutant library from MAP strain A1-157. MAP was cultured 2 and 4 mo post inoculation from intestinal tissues and lymph nodes. Quantitative PCR was used to assess changes in proportions of select gene mutants between intestinal tissues and the inoculum; this method failed to confirm previously described essential genes. After applying filtering parameters, targeted next generation sequencing discovered 42 genes absent from all tissues which are involved in DNA transcription, pathogenesis, metabolism and biosynthesis, and oxidation-reduction reactions. These results provide many potential genes for attenuation of MAP for the creation of candidate vaccine strains.

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advice, and were always there to lend an ear. Watching their successes was inspiring and encouraging.

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DEDICATION

For my dad.

When I was a little girl I watched my dad work hard to provide for me and my 4 brothers and sisters. His determination was evident when he took a job in Florida and moved us all there because 'that was God's plan'. When I was a youth in junior high he started taking night classes at the local university. He managed a full-time job, regularly attended our after-school activities, and his evening classes. He persevered and triumphed because he knew there was something better out there. My dad showed me what passion and persistence looks like. And after many years and lots of late nights and early mornings, he got his masters degree. I still remember sitting in the stadium when he walked across the stage and raised his hand up with degree in hand, smiling. We were all so proud. God had a plan. And we moved to our next great adventure and my Dad's dream job in Delaware. His drive and determination to succeed paid off.

Watching my dad survive cancer the first time was the most stressful and difficult time of my life. But no matter how bad he looked or even when he couldn't speak from the radiation treatment, he smiled, gave a thumbs up, and kept on going. He was inspiring! Despite the pain, he had a positive outlook. God had a plan. And I kept going too. I kept persevering, finished college, got a few different jobs, and eventually got my first masters degree.

You see, despite what happens next, I know that God has a plan. Dad's cancer has come back, and he's still as positive and inspiring as ever! Out of everything my dad taught me, it is most important to have faith and keep going. Because it is with determination, perseverance, and passion that all things are possible—even a PhD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND ILLUSTRATIONS

LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

Symbol	Definition
A1-157L	A1-157 mutant library
ATP	Adenosine triphosphate
BHI	Brain-heart infusion
CFT	Caudal-fold skin test
CFU	Colony forming unit
Ct	Cycle threshold
CWC	Cell wall competent
CWD	Cell wall deficient
DDA	Dimethydioctadecyl ammonium bromide
HPC	Hexadecylpyridinium chloride
Hsp	Heat-shock proteins
ICL	Isocitrate Lyase
IFN-γ	Interferon-gamma
IG	Intergenic region
IgG	Immunoglobulin G
IL	Interleukin
ILN	Ileal lymph nodes
JAK-STAT	Janus activated kinases-signal transducer and activator of transcription
JD	Johne's disease
JDIP	Johne's disease integrated program
JLN	Jejunal lymph nodes
LAV	Live attenuated vaccine
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
NGS	Next generation sequencing
ODNs	Oligodeoxynucleotides
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PE	Proline-Glutamic acid
PHA	Pokeweed mitogen and phytohemagglutinin
PI	Post inoculation
PI3P	phosphatidylinositol 3-phosphate
PPD	Purified protein derivatives
PPE	Proline-Proline-Glutamic acid
PRRS	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
SAM	Aligned Sequence alignment/map
SOD	Superoxide dismutase
TA site	Thymine Adenine dinucleotide site
T_h1	Type 1 T-helper cell

TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
Tn-Seq	Transposon Sequencing
VSRS	Veterinary Science Research Station
wt	Wild type
PBMCs	Peripheral blood mononuclear cells

OBJECTIVES

1) To use a saturated library of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) transposon mutants and targeted next generation sequencing to determine genes essential for MAP to survive in intestinal tissues and lymph nodes of dairy calves.
2) To explore relative qunatification as a method for determining if specific genes of MAP previously reported as essential in mice are also essential in infected dairy calves.

HYPOTHESES

Virulence genes enable MAP to chronically infect the intestinal tissue of dairy calves. To determine which genes are essential for MAP infection to become chronic in dairy calves, a library of mutant bacteria will be created using a transposon or jumping gene which randomly inserts itself into the genome indiscriminately disrupting genes, including putative virulence genes. Following incubation in calves the output pool of bacteria will be compared to the input pool using targeted next generation sequencing techniques.

The sequencing results will reveal an underrepresentation of mutants with disrupted virulence genes by identifying mutants that were cultured from the harvested tissues and comparing them to the inoculum. In addition, the proportion of previously identified essential MAP genes with transposon insertions in the pool is expected to decrease in the harvested tissues in relation to their proportion in the inoculum. Similarly, the proportions of nonessential genes are expected to remain unchanged between the inoculum and the tissues.

INTRODUCTION

Johne's Disease

Johne's disease (JD) is a chronic intestinal disease that negatively affects the dairy cattle industry worldwide. The causative agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and animals with JD exhibit symptoms such as chronic diarrhea, weight loss, decreased milk production, and eventually death. MAP can infect many animals such as wild ungulates, rabbits, mice, and even some carnivores (Carta et al., 2013), however, domestic dairy cattle, sheep, and goats are the primary hosts (Tiwari et al., 2006, Carta et al., 2013).

The dairy industry suffers direct losses stemming from reduced milk production, early mortality, premature culling of infected animals, and decreased carcass value (McKenna et al., 2006). Reduced annual adjusted value of production in MAP-positive herds is mostly attributed to reduced milk production. A herd with a high number of clinical JD-positive cows could see losses greater than 700 kg of milk production per cow per yr (Ott et al., 1999). Loss of cattle is the second greatest cost to the producer. A herd with high numbers of clinical JD-positive cows could experience 22% more mortalities due to culling compared to a MAP-negative farm. In addition to a higher rate of culling, these cows tend to have poorer body conditions and subsequently lower market prices (average difference of $154 US between poor body condition vs. normal body condition). Higher losses increase cow replacement costs resulting, on average, in an additional $32 US per cow (Ott et al., 1999). It is estimated that JD costs Canadian dairy farmers $2472 annually for a 50-cow herd (Chi et al., 2002), whereas the United States estimates a loss of $220 to $250 million annually (Ott et al., 1999).

Mycobacterium avium subsp. *paratuberculosis*

Infection with MAP most often occurs within the first 6 mo of life (Windsor and Whittington, 2010); however, calves can be infected with MAP up to at least 1 yr of age (Mortier et al., 2013). Additionally, infections at an even older age have been reported (Windsor and Whittington, 2010). Calves often do not develop clinical symptoms until they are 2 to 5 yr of age, intermittently shedding MAP in their feces during this subclinical infection stage (Stabel, 1998). Cattle contract the bacteria primarily through a fecal-oral route. However, MAP can also be transmitted in colostrum and milk (Garcia and Shalloo, 2015) and, although a proportion is killed, MAP can survive high temperature, short-time pasteurization (Grant et al., 2002, Ellingson et al., 2005, Shankar et al., 2010, Hammer et al., 2014). Based on the culture of environmental samples, 68% of Alberta dairy farms and 76% of Saskatchewan dairy farms were positive for MAP (Wolf et al., 2014a). In addition, large farms (> 200 cows) are at a higher risk of contracting MAP. In the United States 70% of dairy herds are MAP-positive based on culturing environmental samples (Lombard et al., 2006).

MAP is incredibly successful at surviving adverse conditions. It can survive extreme cold (Richards, 1981) and hot temperatures as indicated by its resistance to pasteurization (Grant et al., 2002). It can survive in water for up to 48 wk, in fecal material for 55 wk, and on the grass for 24 wk (Whittington et al., 2004, Whittington et al., 2005). In addition, it can survive in a highly alkaline cattle dip (pH 12.4) used for the prevention of ticks for 2 wk (Eamens et al., 2001). MAP can also thrive and replicate within free-living amoebas (Whan et al., 2006), and research has even shown that MAP can be internalized by plants though their ability to survive is not understood (Kaevska et al., 2014).

The ability for MAP to survive in extreme conditions can be attributed to its physical characteristics. MAP is a rod-shaped, gram-positive, acid-fast (resists decolorization by acidified alcohol) bacterium. It possesses a thick, waxy cell wall comprised of 60% lipids and polysaccharides which creates hydrophobicity and contributes to its resistance to chemicals and physical processes. However, this lipid-rich cell wall restricts nutrient uptake thus contributing to its extremely slow growth (Rowe and Grant, 2006). MAP has a generation time of approximately 20 h, making it the slowest growing of all the *Mycobacterium* species (Lambrecht et al., 1988). In addition, *Mycobacterium* species characterized as slow-growing (generation times greater than 5 h and more than 7 d for visible colonies to appear on solid media) tend to be pathogenic while fast-growing *Mycobacterium* species are usually non-pathogenic (generation time up to 5 hours and less than 7 days for visible colonies to appear on solid media, Lewin and Sharbati-Tehrani, 2005). Growing MAP colonies on solid media can take on average 6-10 wk depending on the strain; more difficult strains may require up to 6 mo to appear (Whittington et al., 1999, Rowe and Grant, 2006). The ability for MAP to sequester inside of macrophages in combination with its thick cell wall make it highly resistant to antimicrobials. Currently, the best course of action for infected cattle is culling them from the herd to reduce transmission.

Pathogenesis of MAP Infection

MAP is transmitted mainly through a fecal-oral route. Once ingested MAP travels through the gastrointestinal tract. While the ileum is considered the primary route of infection, an early oral inoculation trial suggested that the tonsillar crypts may also serve as a site of infection (Payne and Rankin, 1961) and provide the first opportunity for MAP to invade the tissue of the host (Waters et al., 2003). It is also likely that MAP can infect the jejunal region of the small

intestine though to a lesser degree than the ileum (Ponnusamy et al., 2013, Arsenault et al., 2014). The mechanism by which MAP invades the tissue is preferentially through the microfold (M) cells of the Peyer's patches although enterocytes may also play an important role in the internalization of MAP (Momotani et al., 1988, Pott et al., 2009). As MAP passes through the digestive tract, fibronectin attachment protein is activated on its cell well (Bannantine and Bermudez, 2013). This promotes opsonisation by fibronectin, a glycoprotein, which links MAP to the M cells of the epithelial layer.

Uptake of MAP into the intestinal epithelial layer takes as little as 30 min upon contact (Arsenault et al., 2014). MAP transverses the epithelium into the submucosa where it is specifically taken up by sub-epithelial macrophages which contain several families of receptors involved in mycobacteria uptake (i.e., complement receptors, immunoglobulin receptors, and mannose receptors). MAP utilizes different routes of uptake by macrophages which result in unique patterns of cytokine secretion which may enhance its survival in the host (Guirado et al., 2013). MAP opsonized with serum from cattle increases uptake and adherence by macrophages (Hostetter et al., 2005), however, activation of complement receptors reduced macrophage activation which may allow MAP to evade host defenses (Premanandan et al., 2009). MAP establishes a persistent infection within the macrophages of the small intestine. Immune cells meant to protect the host from invading pathogens are instead manipulated by MAP to provide a safe haven from the host's immune response.

MAP utilizes many different mechanisms to evade the host's immune system and avoid being phagocytised by the macrophage in which it resides. The phagosome, a compartment within the macrophage that contains phagocytosed particles, fuses with lysosomes which contain enzymes essential for the destruction of the particles forming a phagolysosome (Kuby et al.,

2007). Some bacteria, once internalized by macrophages, use mechanisms to prevent the phagolysosome fusion. Preventing the fusion of the phagolysosome is a critical mechanism utilized by MAP. Only live MAP is capable of blocking the phagolysosome formation (Cheville et al., 2001). It is suggested that a mycobacterial sulfolipid is responsible for integrating into the phagolysosome membrane and disrupting its function thus impairing the fusion (Karakousis et al., 2004).

In addition, mycobacteria secrete a lipid phosphatase called SapM that hydrolyzes phosphatidylinositol 3-phosphate (PI3P) which is essential for membrane trafficking and phagosomal maturation. PI3P-binding proteins perform many essential duties for the maintenance and integrity of the phagosome such as delivering internalized plasma membrane receptors to the late endosomes, terminating signaling events within late endosomes, and early endosomal fusion (Vergne et al., 2005). The MAP genome contains a very similar amino acid sequence to SapM, and it is likely that a similar role to disrupt PI3P is essential for its survival in macrophages (Rowe and Grant, 2006). A previous study showed that *Mycobacterium tuberculosis* releases mitogen activated protein kinase (MAPK-p38) which blocks specific proteins from associating with late endosomes; blocking MAPK-p38 results in phagolysosome acidification. This suggests a similar function may also be important for MAP survival (Fratti et al., 2003).

To prevent the formation of the phagolysosome MAP employs additional strategies to reduce how responsive macrophages are to MAP infection. Pattern recognition receptors (PRRs) play an essential role in innate immunity in identifying pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are a major type of PRR that are essential for host immune defense against mycobacteria (Kuby et al., 2007). In fact, mutations in TLRs are the prime

reason for reduced pathogen recognition. Mutations repress the innate immune system response and can also leave individuals with greater susceptibility to fungal infections and mycobacterial infections such as leprosy and tuberculosis (Mucha et al., 2009). Some TLRs are important for initiating an immune response, however, others are activated to decrease or suppress an immune response. MAP takes advantage of both of these types of TLRs to enhance its survival in the host.

TLR-9 recognizes and binds to bacterial DNA with unmethylated CpG motifs and activate immune cells to produce cytokines, thereby enhancing the immune response to invading bacteria (Kuby et al., 2007). Mice treated with CpG Oligodeoxynucleotides (ODNs) at the same time as they were inoculated with *M. tuberculosis* were able to reduce their mycobacterial load up to 5 wk after infection. In addition, treatment with CpG ODNs was associated with increased IFN-γ production (Juffermans et al., 2002). This suggests that the ability for the immune system to recognize CpGs by TLR-9 is essential for controlling a mycobacterial infection. However, despite maintaining high TLR-9 expression, bovine monocytes infected with MAP were able to inhibit TLR-9-mediated responses by blocking downstream signaling. While this mechanism is still relatively unknown, MAP is able to divert the TLR-9 signal through the Pyk2 pathway which effectively prevents the clearance of MAP from the monocytes (Rowe and Grant, 2006).

Once the phagolysosome is formed, reactive oxygen intermediates (ROIs) enhance the killing of bacteria. These ROIs which include superoxide anion, hydrogen peroxide, and hydroxyl free radicals are associated with killing mycobacteria (Karakousis et al., 2004, Roca and Ramakrishnan, 2013). However, there is some controversy over their role in MAP infection. Very little ROI is produced by bovine monocytes in response to MAP infection (Zurbrick et al., 1988). This suggests that perhaps ROIs are not effective against MAP bacteria and monocytes

are not stimulated to produce them. On the other hand, MAP has the ability to secrete superoxide dismutase which breaks down ROIs into hydrogen peroxide and molecular oxygen which may protect MAP (Liu et al., 2001). Therefore, it is possible that MAP inhibits the production of ROIs to neutralize the threat.

The phagolysosome also produces reactive nitrogen intermediates (RNIs) against mycobacteria. Nitric oxide synthase is a tightly regulated enzyme responsible for the production of RNIs. Nitric oxide is a highly reactive free radical capable of reacting with other nitrogen species to form RNIs (Davis et al., 2007). Like ROIs, there is some controversy regarding their contribution to killing mycobacterial species. MAP-infected bovine monocytes increase production of nitric oxide in response to IFN-γ. However, the amount of nitric oxide produced by the phagolysosome is considered insufficient to effectively destroy MAP (Zhao et al., 1997). *Mycobacterium tuberculosis* is sensitive to the generation of nitric oxide and inhibits nitric oxide synthase suggesting that RNIs are a potential threat to mycobacteria (Davis et al., 2007).

Interferon gamma (IFN-γ) is a cytokine that is critical for both the innate and adaptive immune responses to many pathogens. It is produced mainly by T-cells and natural killer cells. IFN-γ is a particularly important cytokine for the activation of macrophages, maintaining chronic inflammation, and it increases the expression of MHC protein complexes on the surface of cells to enhance antigen presentation (Kuby et al., 2007). IFN-γ is also essential for responding to an intracellular infection with mycobacteria. In fact, IFN-γ knockout mice are more susceptible to low doses of *M. tuberculosis.* Mice exhibit a widespread infection and significant tissue destruction which indicates the importance of IFN-γ for maintaining and clearing a mycobacterial infection (Cooper et al., 1993). In addition, humans with mutations in the IFN-γ-producing gene have inhibited IFN-γ production, and they are specifically vulnerable to

mycobacterial infections (Dupuis et al., 2000). Cattle exhibiting subclinical symptoms of MAP infection produce more IFN-γ than either uninfected or clinical cattle which suggests that IFN-γ plays a very important role in controlling MAP infection (Sweeney et al., 1998).

IFN-γ activates macrophages via the Janus Activated Kinases-Signal Transducer and Activator of Transcription (JAK-STAT) pathway. First the cytokine binds to a specific receptor which activates a JAK associated with the receptor. Then JAK autophosphorylates and also phosphorylates STAT, activating this transcription factor. This induces a conformational change in STAT allowing it to translocate into the nucleus and activate specific genes (Kuby et al., 2007).

While the host is actively producing IFN-γ to clear the intracellular pathogen, MAP blocks the ability for the macrophage to be activated by the cytokine. MAP has the ability to block signaling associated with the IFN-γ receptor (Arsenault et al., 2012). Bovine monocytes exposed to MAP do not produce similar patterns of phosphorylation consistent with the JAK-STAT pathway that is observed in monocytes unexposed to MAP. Also, exposing the MAP-infected monocytes to exogenous IFN-γ still does not produce peptide phosphorylation consistent with activating the JAK-STAT pathway suggesting that MAP produces a mechanism to block the pathway and not prevent IFN-γ production. It was discovered that MAP stimulates the production of genes SOCS1 and SOCS3 which down regulate the IFN-γ receptor (Arsenault et al., 2012).

It appears that the production of IFN-γ is imperative for the host to respond to a mycobacterial infection. IFN-γ is produced throughout the duration of a MAP infection and is detectable earlier in calves inoculated with a high dose (Mortier et al., 2014c). Pre-treating

macrophages to IFN-γ prior to exposing them to MAP can also enhance clearance of the pathogen, however, once MAP has established itself within the macrophages, treating with IFN-γ is much less effective at clearance (Denis et al., 1990, Bonecini-Almeida et al., 1998). This further suggests that MAP is unable to inhibit the production of IFN-γ but rather the signaling pathway in which it stimulates macrophages to respond to the intracellular infection. Thus, it is very important for the immune system to be stimulated to produce IFN-γ before MAP has the opportunity to take up residence in the macrophages. It is, therefore, vital that a vaccine be administered prior to MAP infection to have the greatest impact on the immune system responses.

MAP utilizes the anti-inflammatory cytokine IL-10 to further evade the host's immune system. IL-10 acts by reducing the expression of IL-12, a pro-inflammatory cytokine that stimulates the differentiation of T-cells into Th1 cells. IL-12 also stimulates the production of IFN-γ, tumor necrosis factor-alpha (TNF-α), and reduces IL-4 which suppresses IFN-γ (Kuby et al., 2007). IL-10 also decreases antigen presentation by macrophages and dendritic cells and inhibits the production of other pro-inflammatory cytokines (Borutaite and Brown, 2003, Kuby et al., 2007). Neutralizing IL-10 produced by macrophages results in an increase of expression of TNF-α, IL-12, acidification of phagosomes, apoptosis of macrophages, and the production of nitric oxide when infected with MAP (Borutaite and Brown, 2003). In addition, 57% of the bacteria were killed within the first 96 h post infection. The mechanisms by which MAP induces IL-10 secretion is currently not known. This knowledge gap may have insight into the further development of a vaccine to prevent persistent infection.

Vaccine Development

Developing a vaccine that is effective at controlling or eradicating paratuberculosis from the dairy industry is highly desirable. The first vaccine against MAP was developed in 1926 and consisted of a live nonvirulent strain of MAP suspended in olive oil, liquid paraffin, and pumice powder as an adjuvant (Vallée and Rinjard, 1926, Rosseels and Huygen, 2008). Extensive progress has since been made in vaccine research and an array of strategies to inoculate livestock against MAP have been developed. Potential strategies include the development of whole-cell killed vaccines, live-attenuated strains, protein-based subunit vaccines, and also integration of MAP genes in viruses or plasmids (Rosseels and Huygen, 2008, Lamont et al., 2014). Earlier strategies demonstrated that revaccination is not recommended and may cause cattle (Stuart, 1965) and sheep (Gilmour and Angus, 1973) to develop gross lesions and clinical symptoms. Thus far, the focus of MAP vaccine administration has been restricted to young animals namely due to the nature of MAP infection and the increased susceptibility of young animals. Also, little research explores vaccinating older animals (Bastida and Juste, 2011).

Whole-Cell Killed Vaccines

The currently available vaccines for the prevention of JD are inherently flawed. Whole-cell killed vaccines do not provide enough protection to the host nor do they prevent the spread of MAP. Mycopar (Boehringer Ingelheim, Ridgefield, CT, USA) is the only vaccine licensed for use in the United States; no vaccines are approved for use in Canada. Mycopar is manufactured with Strain 18 which is actually *M. avium avium* passed down through labs and misidentified for decades (Chiodini, 1993). The whole-cell killed vaccine Gudair (Zoetis, Silverwater, Australia) is approved for use in Europe for the control of JD in sheep and goats. It has shown some

promise as demonstrated in a long-term study of Merino sheep resulting in a 90% reduction of deaths due to JD and reduced fecal shedding (Reddacliff et al., 2006). Other whole-cell killed vaccines have been produced, but they all share some common deficiencies, mainly, that they do not prevent infection nor eliminate shedding of MAP in the feces (Kalis et al., 2001, Emery and Whittington, 2004). Additionally, a large granuloma can develop at the injection site; trauma to this region can cause infection or abscesses to form and they reduce carcass quality resulting in a lower profit at slaughter. Furthermore, the vaccines are cross-reactive with the caudal-fold skin test (CFT) for *Mycobacterium bovis,* the causative agent of bovine tuberculosis, which can produce a false-positive result when animals are tested to exclude *M. bovis* infection (Stabel, 1998).

A whole-cell killed vaccine consisting of a field isolate of MAP was paired with an immune system stimulating adjuvant recombinant IL-12. IL-12 has been implicated as a key cytokine essential for the initiating and maintaining T_h1 cell responses (O'Garra and Arai, 2000). Compared to Mycopar a significant reduction in tissue colonization was observed after challenge, however, its effect on IFN-γ production and overall mycobacterial load was not statistically significant from Mycopar (Uzonna et al., 2003).

Cell-wall deficient (CWD) forms of MAP, also called spheroplasts, have been implicated as the possible cause of Crohn's disease in some patients (Chiodini et al., 1986, Hulten et al., 2000, Chamberlin et al., 2001). Isolating and maintaining CWD MAP from tissues is very difficult; the basis for detection of CWD MAP in tissue is through *in situ* hybridization and subsequent PCR for the insertion sequence IS900 found in MAP (Jeyanathan et al., 2006). However, culturing MAP and treating it chemically can produce a high number of CWD MAP in liquid culture (Hines and Styer, 2003). A heat-killed CWD MAP was compared to cell-wall

competent (CWC) MAP in two different adjuvants, alum and QS21 (saponin), in a challenge trial in goat kids. All four injections resulted in a persistent large nodule at the injection site. The CWC-QS21 vaccine resulted in the least amount of fecal shedding and the lowest number of lesions. However, all goat kids continued to shed MAP in their feces and the vaccines did not produce an efficient level of protection suggesting that CWD MAP are not a viable option for an effective vaccine (Hines et al., 2007b).

A significant impediment for utilizing a whole-cell killed vaccine to combat JD is the need to develop a vaccine with a marker to distinguish it from infected animals. The term DIVA, Differentiating Infected from Vaccinated Individuals, was coined in 1999 as a type of vaccine to improve diagnosing animals that are vaccinated versus infected with a specific disease (van Oirschot, 1999). A DIVA vaccine when used in conjunction with its complementary diagnostic tests will specifically generate an immune response in the host that differs from an infected animal. The vaccine is produced by omitting a specific immunogenic protein or negative marker. The accompanying diagnostic test measures the host's antibody response to the omitted protein thus identifying an infected animal (van Oirschot et al., 1986). In addition, a foreign antigenic protein can be added as a positive marker which may be a useful tool for differentiating vaccinated animals from infected animals.

Subunit Vaccines

Bacterial cell fractions or recombinant protein antigens have also been investigated as candidates for a MAP subunit vaccine. Kathaperumal et al. (2008) demonstrated that recombinant antigens 85A and 85B complex antigens and superoxide dismutase (SOD) protected calves against a MAP challenge, and also that MAP74F, a fusion polypeptide, protected mice

against a MAP infection (Chen et al., 2008). The research group used an inoculum consisting of the four recombinant antigens (85A, 85B, SOD, and Map74F) and an adjuvant (dimethydioctadecyl ammonium bromide (DDA)), shown to be effective at enhancing cellular immunity in tuberculosis subunit vaccines (Dietrich et al., 2007), to subcutaneously immunize goats prior to challenging with MAP. The animals were boostered and challenged again 3 wk after the initial vaccination. At six wk from administration of the primary vaccine goats produced an IFN-γ response specific to the antigens in the vaccine that peaked at 10 wk post inoculation (PI). Also, significant lymphocyte proliferation was observed throughout the duration of the trial. In addition, of the 23 tissues collected from the goats at necropsy, MAP was cultured from only 1 out of all 8 goats in the treatment group; this was substantially less than the unvaccinated control group (Kathaperumal et al., 2009).

While this particular experiment indicates that the development of a subunit vaccine for the prevention of JD seems promising, there are disadvantages. For one, this particular vaccine has only been tested in goats, and it is not known if it will perform similarly in dairy cattle. These animals may have different immune responses to the vaccine. Gudair is fairly successful in reducing fecal shedding in sheep and goats but the manufacturer has not published data for its efficacy in cattle (Zoetis, Australia; Reddacliff et al., 2006). Also, the authors for this study did not report if the vaccine was effective at reducing fecal shedding which is a very important concern for controlling the spread of MAP between cattle. The authors also expressed that the adjuvant DDA only evoked a mild increase in lymphocyte and IFN-γ production compared to no adjuvant. They suggest using more costly adjuvants to boost the immune response, but increasing the overall cost of a vaccine may be prohibitive to producers (Kathaperumal et al., 2009).

Heat-shock proteins (Hsp) are a family of highly conserved proteins that are expressed in response to a stressful environment. These proteins have been shown to be highly immunogenic antigens in many mycobacterial species including MAP (Koets et al., 2001), capable of inducing a cell-mediated immune response (Koets et al., 1999). This same research group developed a vaccine consisting of recombinant Hsp70 paired with the adjuvant DDA (Hsp70/DDA) to inoculate and booster calves prior to challenging with MAP-infected feces (Koets et al., 2006). Calves belonging to 1 of 4 treatment groups (negative control, immunized and unchallenged, inoculated with no vaccine, and immunized and inoculated) were followed for two years. Calves in all four treatment groups displayed a similar IFN-γ response, however, the two groups receiving both the primary immunization and the booster, exhibited a distinct surge in IgG expression. While there was a reduction in fecal shedding compared to the inoculated and unvaccinated group, 5 out of the 10 calves shed MAP actively at least once.

In a later study on calf-to-calf transmission regarding vaccinated and challenged calves exposed to contact calves (Santema et al., 2012), the Hsp70/DDA vaccine proved to be unable to reduce fecal shedding; however, the calves did experience a long latency phase where they had a very low frequency of shedding for approximately 200 d before continuing to shed. Also, tissue colonization of MAP in calves vaccinated did not differ from calves that were not vaccinated demonstrating further that the vaccine did not prevent MAP infection. However, the study did demonstrate that vaccinated and challenged calves placed in pens with contact calves resulted in little transmission to the contact calves when compared to contact calves housed with inoculated only calves. This indicates that vaccination may have significantly reduced fecal shedding during a time when calves are most susceptible to infection and also prevented calves from developing into super shedders thus reducing on-farm transmission.

This same research group set out to determine the primary immune response in calves to the Hsp70/DDA vaccine by lymph vessel cannulation from the prescapular lymph node near the site of vaccination (Vrieling et al., 2013). Unfortunately, this demonstrated that the Hsp70/DDA vaccine evoked an abundance of IgG antibody production and the proliferation of memory B cells, but did not result in a T_h1-type immune response nor did it stimulate the production of IFN-γ which have been shown to be essential in protecting against mycobacterial infections (van Crevel et al., 2002). Based on the limited T_h1-type immune response, the inability to significantly reduce or eliminate fecal shedding, and the inability to prevent MAP from infecting the tissue the Hsp70/DDA vaccine is not a viable candidate for the prevention of MAP infection in calves.

Many MAP researchers have focused their attention on developing a live-attenuated vaccine because it is more likely to generate a T_h1-type immune response which is considered essential for protecting the host against mycobacterial infections (Stabel, 2000, Hostetter et al., 2002). In addition, production of subunit vaccines may prove to be too expensive to produce and the cost to the livestock production industry would be unreasonable. A less expensive option could be the development of a DNA vaccine. There have been several publications evaluating the efficacy of DNA vaccines (Huntley et al., 2005, Park et al., 2008, Romano and Huygen, 2009, Roupie et al., 2012), however, they have all been performed in mice and resulted in only modest protection from MAP challenge.

The Johne's Disease Integrated Program (JDIP) established standardized methods for evaluating 22 vaccine strain candidates, all of which were live-attenuated (Bannantine et al., 2014). While all of the investigated strains failed to induce a protective immune response, the authors suggested in their discussion that funding and focus should be placed on the development

of a live-attenuated vaccine for the prevention of JD. While this trial established a framework for vaccine trial design, the authors concluded that though mice and macrophage experiments were cost-effective, future investigations for evaluating MAP vaccine candidates would be best performed in the primary host.

Live-attenuated Vaccines

Recent studies have published promising results that demonstrate live, attenuated vaccines (LAV) may provide more robust protection compared to killed vaccines. For example, a live, attenuated strain of MAP with a *leuD* mutation induced a prolonged IFN-γ response, proliferation of T-cells, and induced higher levels of pro-inflammatory cytokines in vaccinated goats that are challenged with a clinical MAP strain (Faisal et al., 2013). The *leuD* gene, discovered in *M. tuberculosis*, encodes an essential enzyme for leucine synthesis. While vaccination with the *leuD* mutant was able to reduce colonization of the intestinal tissue, it did not prevent infection or fecal shedding. Preventing fecal shedding is vital for controlling the spread of JD and reducing or eliminating the presence of MAP on farms. This suggests that a *leuD* mutant may not be a good target for JD prevention since it does not stimulate the immune system to rid the host of infection.

Another example of a potential LAV knocked out the gene *relA*, a global regulator, from MAP strain K-10. An *ex vivo* experiment involving the infection of bovine monocyte-derived macrophages demonstrated a significant reduction in survival (12.5 ± 6.3%) compared to wildtype K-10 (29.4 ± 3.6%) following 6 d after initial infection. In addition, following ileal cannulation of an infectious dose of MAP, no *relA* mutant bacteria were detected in the intestinal tissues and lymph nodes of dairy calves compared to wildtype K-10 3 mo post infection. Based

on the successful initial results, a 2-mo follow-up pilot challenge trial in goats was also performed. Not only was the *relA* mutant cleared from the tissue, but a very minimal number of colony forming units (CFUs) from the challenged wildtype K-10 strain were recovered compared to inoculation with the K-10 strain (Park et al., 2011). These promising results suggested that the *relA* mutant may be a very good candidate for a MAP vaccine. Therefore, a follow up challenge trial in dairy calves was also performed. Calves were vaccinated with the *relA* mutant at 6 d of age and challenged with MAP K-10 1 mo later. Calves were euthanized 3 mo post vaccination. While the *relA* mutant was cleared from the intestinal and associated tissues collected from the calves, it did not prevent the colonization of the challenge strain. Bacterial loads were significantly reduced in the tissues compared to the K-10 wildtype controls. The researchers did not report fecal shedding of MAP; therefore, it is unknown whether or not vaccination with *relA* was able to reduce transmission (Park et al., 2014). These results suggest that the *relA* mutant should continue to be considered as a possible vaccine candidate though more information about how it reduces infection and potential fecal shedding should be investigated.

Vaccine Development Methods

Developing a more effective vaccine to prevent JD is crucial for reducing the prevalence of MAP infection. Previously developed vaccines are ineffective at inducing a protective immune response and preventing the spread of MAP. A more beneficial vaccine requires specific elements to overcome these shortcomings. The attenuated vaccine should be able to prevent long-term infection in dairy calves. It must be strong enough to trigger an immune response to prevent infection and eliminate fecal shedding which would reduce transmission. If the vaccine could be administered orally it would eliminate the inflammatory response that occurs at the

injection site. It should also be able to protect calves against many different strains of MAP and contain markers to differentiate the vaccine strain from wildtype MAP infections and not interfere with *M. bovis* testing.

Previous attempts to investigate potential genes for attenuation of MAP have largely been unsuccessful. The framework suggested by JDIP begins by studying attenuated strains of MAP in macrophages and then tissue colonization in mice. Strong potential candidates advance to a challenge trial in goats to evaluate their ability to reduce fecal shedding and colonize tissues (Bannantine et al., 2014). Infecting mice, while less expensive, provides an inadequate environment for the incubation of MAP since mice have different digestive tracts as well as a very different immune system from cattle. Many LAV that appear to elicit promising results in mice (i.e. little to no colonization in tissues, elicit a strong immune response) have either shown little success in ruminants or never made it to a clinical infection trial (Mullerad et al., 2002, Scandurra et al., 2010, Chandra et al., 2012). While this framework establishes a standard method for evaluating attenuated vaccine strains, it is costly and time consuming and has yielded few promising results.

In lieu of evaluating one mutant strain at a time, Wang et al. (2014) used a novel method for evaluating thousands of MAP mutants concurrently using a mouse model. A library of mutants was developed using a MycoMarT7 phagemid containing a mariner transposon which inserts randomly at TA dinucleotide sites in the MAP genome creating disrupted genes (Siegrist and Rubin, 2009). Mice were injected intraperitoneally and incubated for 1 mo. Spleens were collected and targeted whole genome sequencing was performed to evaluate disrupted virulence genes that were no longer present in the tissue (Wang et al., 2014). The researchers were able to produce mutants representing 56% or 2443 genes of the MAP genome prior to inoculating their

mice. The researchers identified 29 genes absent from the spleens of each of the four mice which represent possible candidate genes for developing a knockout strain of MAP. However, these results were based on a single TA dinucleotide site represented in the inoculum and did not consider all of the disrupted TA sites for each gene. In addition, 10 of the listed genes had less than 100 copies in the inoculum; lower representation in the input pool may affect the number of copies present in the output pool suggesting specious results. When the raw data from the publication was evaluated for all of the disrupted TA sites for each gene, there were no genes in which the majority of TA sites were disrupted in the inoculum, and the gene was absent from all of the mice. This could be due to low saturation of TA sites represented in the inoculum. The inoculum was only comprised of one library of mutants; it was suggested by the researchers that producing multiple libraries may increase the number of TA sites represented and thus yield more definitive results. While this method represents an important scientific achievement, it did not identify essential genes to target for an attenuated vaccine in cattle.

Tn-Seq

Targeted whole genome sequencing is a multifaceted technique to sequence specific regions or an entire genome. The method employed by Wang et al, (2014) was a procedure called Tn-seq or transposon sequencing. Tn-seq is based on assembling a library from a particular bacterium which is saturated with a Mariner transposon insertion (van Opijnen et al., 2009). Using a cut and paste method, the transposase enzyme recognizes the inverted repeat regions at the end of the Mariner transposon, identifies the targeted sequence, makes a double-stranded break, and inserts the transposon into the targeted TA site (van Opijnen and Camilli, 2013). Insertional mutagenesis allows researchers to directly link an observed phenotype such as

survival to a genotype.

Once the library is created, the organism is exposed to a specific treatment and changes to the frequency of each mutant with a Mariner transposon insertion are determined by sequencing the flanking regions in parallel. The change in frequency of each mutant before and after treatment is calculated to determine the fitness that each gene has on the ability for the organism to survive (van Opijnen et al., 2009). Fitness of every insertion point in a genome can be determined as a quantitative measure of the rate of growth or survival.

Utilizing dairy calves as the model for studying MAP survival ensures that we begin with the primary host. Tn-seq will be used to develop a library or pool of mutants using a mariner transposon (Murry et al., 2008). Thousands of potential mutants will be be evaluated in massive parallel sequencing for their role in MAP survival. Targeted next generation sequencing (NGS) will reveal the disrupted virulence genes that were unable to survive in the host tissue. These genes will serve as candidates for a live vaccine that can be cleared from the host. This method addresses the translational problems experienced in the mouse model while screening thousands of potential genes. Identification of essential genes and subsequently knocking them out could drive the development of a more successful vaccine capable of inhibiting MAP from establishing an infection and preventing fecal shedding ultimately reducing the prevalence of MAP infection and JD.

MATERIALS AND METHODS

Calves

Sixteen Holstein-Friesian bull calves were purchased from 6 dairy farms in Alberta, Canada. Farms selected had < 5% herd prevalence of MAP infection based on serum sampling recorded for the Alberta Johne's Disease Initiative (Calgary, AB, Canada; Wolf et al., 2014b). Contact was avoided between calf and dam and the calves were collected within 12 h after birth. In addition, producers were responsible for spraying the umbilicus with an iodine solution such as Betadine or Povidone diluted in water (1 part iodine to 10 parts of water). The calves were fed 3 L of rehydrated colostrum powder (2 pouches, Headstart, Saskatoon Colostrum Company, Saskatoon, SK, Canada) within 6 h after birth.

Health, Nutrition, and Husbandry

Newborn calves were transported to the research barn located at the Veterinary Science Research Station (VSRS) at the University of Calgary. To ensure calves recieved adequate nutrition and antibodies, they were bottle-fed an additional 1.5 L of rehydrated colostrum powder (1 pouch, Calf's Choice Total, Saskatoon Colostrum Company, Saskatoon, SK, Canada) upon arrival. The calves were fed milk replacer, weaned at 7 wk of age to a calf starter grain, and fed ad libitum high quality hay and water.

The calves were housed in a biosafety level 2 facility for the duration of the trial. Calves were individually housed in custom-built pens similar to Mortier et al. (2013, 2014b). Each pen was lined with a waterproof liner to contain all contaminated bedding and manure. To prevent cross-contamination each treatment group was equipped with dedicated boot dips, boots, coveralls, gloves, and tools used to maintain the pens. VSRS personnel were trained in biosafety

protocols to avoid transmitting MAP between pens and outside the facility. Individual calves were monitored and the VSRS personnel recorded their health status daily. Animals were under veterinary care during the length of the trial. The Veterinary Science Animal Care Committee of the University of Calgary approved the animal care protocols for this research trial (VSACC AC13-0060).

Inoculum

A parent strain of MAP was chosen from 182 Canadian isolates. A1-157 is an Alberta isolate that belongs to a dominant clade that contains more than 80% of all Canadian MAP isolates (Ahlstrom et al., 2015). To screen for essential genes involved in pathogenesis of MAP, a library of mutants composed of disrupted genes was created using a transposon as described (Murry et al., 2008). Three independent libraries were produced using the MycoMarT7 phagemid which contains a kanamycin resistance cassette and inserts at TA dinucleotide sites within mycobacterial DNA. The phagemid was titrated using *M. smegmatis* on kanamycin-selected plates. The MycoMarT7 phage was incubated at 30°C with *M. smegmatis* until plaques formed in approximately 3 d.

Using the pelleted wet weight method (Hines et al., 2007a) our lab determined that for an OD_{600} of 1.0 MAP strain A1-157 is approximately 10^8 CFUs. Briefly, each independent library of MAP was grown to an OD_{600} of ~1.0 and 20mL were transduced with ~4 x 10^{10} phages (MOI = 20, Murry et al., 2008). MycomarT7 phage and MAP were incubated at 37°C for 4 h in MP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10mM $MgSO_4$, 2 mM $CaCl_2$). 7H9 medium enriched with Mycobactin J (2g/L), glycerol (0.4%) and OADC (10%) was added to the MP buffer mixture and incubated overnight at 37°C in a shaking incubator. Mutants were spun down,

resuspended in 7H9 medium, and plated on several kanamycin (25μg/mL) selective plates. Approximately 8 wk later, ~100,000 CFUs of kanamycin-resistant colonies were counted and scraped from the plates using a cell scraper (1.8 cm blade, BD Falcon Cell Scraper, Fisher Scientific, Pittsburgh, PA, USA), resuspended in 7H9 medium and vigorously vortexed to break up the clumps. Each library was grown to an OD_{600} of ~1.5, aliquoted into 25% glycerol stocks, and frozen at -80°C.

Trial Design

The 16 calves were randomized by birthdate to 3 different treatment groups. At 2 wk of age, 12 calves were inoculated with the A1-157 (A1-157L) mutant MAP library. Four calves served as controls and were inoculated with the wildtype (wt) A1-157 strain. Calves were inoculated with a very high dose of MAP, 5×10^{10} CFUs, given on 2 consecutive days. Inoculum was quantified using the pelleted wet weight method. One day before the calves were dosed, aliquots of each library were thawed, equally pooled together, and incubated in 7H9 broth enriched with Mycobactin J (2g/L), glycerol (0.4%) and OADC (10%) at 37°C in a shaking incubator. The inoculum was drawn up into a 50 cc syringe and each calf suckled the inoculum completely. 8 calves receiving the mutant library and 2 calves receiving the wildtype strain were euthanized at 2 mo PI; the remaining calves were euthanized at 4 mo PI.

Sampling

Fecal Samples

To confirm that each calf was infected and actively shedding MAP, fecal samples were collected from the rectum of each calf once each month PI for 4 mo. DNA was extracted using the MagMax™ Total Nucleic Acid Isolation kit (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions with some modifications. Briefly, 1 mL of phosphate-buffered saline (PBS) was added to 0.3g of homogenized feces and vortexed. Following centrifugation on a very low speed (100g for 1 min), the supernatant was transferred to a Bead Tube containing lysis buffer. The samples were bead-beaten twice for 5 min on a mini-Beadbeater (Biospec, Bartlesville, OK, USA) and cooled on ice between beatings. Samples were pelleted by centrifugation and the lysate was transferred to a processing plate. Nucleic acid was bound to Nucleic Acid Binding Beads and a 96-well magnetic ring stand was used to capture the beads. Samples were washed twice with each of two Washing Solutions. Elution Buffer was used to capture the DNA in the supernatant separating it from the beads.

Real-time PCR was used to quantify DNA extracted from all of the fecal samples. A PCR protocol was used according to Slana et al. (2008) for IS*900* and F57 with identical primers, probes, and internal amplification control sequences. TaqMan probes were purchased from Biosearch Technologies, Inc. (Novato, CA, USA). Each reaction contained 10 uL of TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), 10 pmol of each primer, 1 pmol of the internal control plasmid, 2 uL of the template. Thermocycler (Bio-Rad C1000 Thermal Cycler, Hercules, CA, USA) conditions were as follows: 50°C for 2 min, denaturation at 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 61°C for 30 sec.

Interferon-gamma Assay

At 2 and 3 mo PI, whole blood samples were collected in heparinized tubes. Samples were transported in an insulated container containing hot water bottles to keep the blood within an optimal temperature range. The IFN-γ release assay was performed within 2 h after collection as described (Mortier et al., 2014c). Briefly, whole blood (1.5 mL) was exposed to 100 µl of purified protein derivatives (PPD) Johnin, Avium, and Bovine (0.3 mg/mL; Canadian Food Inspection Agency, Ottawa, ON, Canada) which were compared to the IFN-γ response generated against the positive controls (phytohemagglutinin (PHA) and pokeweed mitogen (PWA)) (0.3 mg/mL Sigma–Aldrich Canada Co., Oakville, ON, Canada) and the unstimulated or negative control (PBS). All samples were incubated overnight at 37°C in a 5% CO_2 atmosphere and plasma IFN-γ was determined using ELISA BOVIGAM^{TM} (Prionics, La Vista, NE, USA) according to manufacturer's instructions. Interpretations of IFN-γ production was based on a previous method for calculation (Kalis et al., 2003, Mortier et al., 2014c). To correct for variations observed in different plates, %IFN-γ for each PPD stimulant was calculated as follows: %IFN-γ = (PPD – average negative assay control)/(average positive assay control – negative assay control) x 100.

Serum ELISA

Prior to euthanasia, serum samples were collected from the calves that were euthanized at 4 mo PI. Serum samples were analyzed using the Idexx Paratuberculosis AB Test for MAP-specific antibodies (Idexx Laboratories, Inc., Westbrook, ME, US) according to manufacturer's instructions. A sample to positive (S/P) ratio ≥ 0.70 was considered positive and ≤ 0.60 was considered negative.

Necropsies

Calves were euthanized at either 2 or 4 mo PI. Calves were sedated with Rompun (0.2 mg/kg) and euthanized by bolt gun or intravenous injection of barbiturate (1ml/5kg; Euthanyl Forte®, DIN 00241326, Bimeda-MTC Animal Health Inc., ON, Canada). Necropsies were performed immediately. Before and after each necropsy all tables, instruments, and other supplies were cleaned and disinfected. Eight tissues were collected from each calf: the ileum and ileocecal valve, 2 sections from the distal jejunum, all ileal lymph nodes, 2 pools of distal jejunal lymph nodes, inguinal lymph nodes, and spleen. A sterile set of instruments was used for each type of tissue. The spleen and lymph nodes were collected prior to opening the intestinal samples to avoid potential contamination from intestinal contents. Intestines were marked and tied off with zip ties to prevent the intestinal contents from shifting and leaking. Intestinal samples were cut down the side connecting the mesenteric tissue and the contents were washed off the tissue under running water. All tissues were stored in whirl-pak™ bags (Nasco, Fort Atkinson, WI, USA) with PBS and transported to the lab in an insulated container.

The mucosa of the intestinal tissue was scraped and collected using glass microscope slides. After the fat was trimmed and removed from the lymph nodes, a scalpel was used to cut them into very small pieces. Tissues that were not immediately used were stored in whirl-pak™ bags (Nasco, Fort Atkinson, WI, USA) at -80°C.

Bacterial Culturing

Tissues were homogenized used a gentleMACS™ Dissociator (Miltenyi Biotec, Inc., San Diego, CA, USA). Up to 10 g of the scraped intestinal tissue or lymph nodes was collected and

divided into 2 gentleMACS™ M tubes. Tissues were incubated for 1 h in a 0.5% Triton X-100 solution with PBS enriched with 1% Y-30 Emulsion (#A6457, Sigma Aldrich, St. Louis, MO, USA) to prevent foaming. Frozen tissues were treated with the same protocol except that the Y-30 Emulsion was not added to the homogenate. Tissue was homogenized in the gentleMACS™ Dissociator twice using program Protein_01.01. Each tissue was combined in a sterilized bottle and PBS was used to wash out any remaining tissues in the M tubes. Tissues were centrifuged at 7,000 x *g* for 20 min and the supernatant was carefully poured off. The pellet was resuspended in 25 mL of 0.75% hexadecylpyridinium chloride (HPC) in half-strength Brain-Heart Infusion (BHI) and 10 to 12 4mm sterile glass beads. Pellets were vigorously vortexed for 2-5 min to break up the pellet. Tissues were incubated for 3 h at 37°C. Tissues were centrifuged at 7,000 x *g* for 15 min and the supernatant was poured off. The pellets were put into Para-JEM™ AS (Thermo Scientific, Waltham, MA, USA) antibiotic mixture (0.2 mL Para-JEM™ AS (Vancomycin, Nalidixic Acid, and Amphotericin), 1.5 mL full-strength BHI, 1.3 mL ddH_2O) and incubated overnight at 37°C.

The next day 25 mL of PBS was added, the pellets vigorously vortexed to break up the tissue, and centrifuged at 4700g for 15 min. The supernatant was poured off and the tissue resuspended in 25 mL of 0.2 M sucrose. Tissue was centrifuged at a very low speed (200 x *g* for 15 min) to allow the sucrose gradient to separate bacteria from the tissue. The supernatant was carefully pipetted and the sucrose gradient repeated. The supernatant was spun down at 4700 x *g* for 15 min, resuspended in PBS, and plated on several 7H11 kanamycin selective plates with Amphotericin B. The plates were incubated for 8 wk. Serial dilutions were made for all the supernatants in addition to undiluted plating. All the CFUs were counted and recorded. The

plates were scraped using cell scrapers and the bacteria were collected by individual calf tissue and frozen at -80°C.

DNA Extraction

MAP colonies were scraped into 10 mL of 1X TE Buffer with lysozyme (1mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and incubated in a shaking incubator for 2 d at 37°C. Then the samples were incubated for 20 min at 65°C with 10% SDS and Proteinase K (10mg/mL). To separate the polysaccharide-rich cellular membrane of MAP, a CTAB/NaCl solution was added and incubated for 10 min at 65°C. Chloroform:isoamyl alcohol was added and centrifuged for 20 min at 2000 x *g*. The upper phase was transferred to ice cold isopropanol then centrifuged at 10,000 x *g* for 10 min at 4°C. The pellet was resuspended in ice cold 70% ethanol and centrifuged for 10 min at 10,000 x *g* at 4°C. The supernatant was removed and the DNA was resuspended in ddH_2O.

qPCR Quantification of Selected Genes

Real-time or quantitative PCR (qPCR) was utilized to determine if genes could be relatively quantified and evaluated for being essential for infection and survival in dairy calves. DNA extracted from MAP cells from the inoculum and from selected tissue were used for this analysis. Nineteen tissues were selected based on tissue type and number of CFUs isolated on solid media (Table 1).

Cycle thresholds (Ct values) were obtained for each selected gene from both inoculum and selected tissues. The Ct values were compared using a modified delta delta Ct method (Livak

and Schmittgen, 2001) in which the F57 gene served as the reference gene (Poupart et al., 1993, Ellingson et al., 2000, Strommenger et al., 2001).

Four putative essential genes, and 3 putative nonessential genes were selected as determined by published results from Wang et al. (2014). Previously, these mice were intraperitoneally injected with an inoculum of MAP produced using a mariner transposon. Mice were incubated, and the spleens were cultured on solid media for surviving MAP mutants.

The essential genes chosen for this study were selected based on the following parameters: the majority of TA dinucleotide sites in the genes must have a low output to input ratio (~0.0), there must be greater than 1,000 reads of each mutated TA site in the inoculum, and the genes must be less than 1,500 bp long. Nonessential genes must have the majority of mutant TA sites represented with an output to input ratio of ~1.0, must also have greater than 1,000 reads of each mutated TA site in the inoculum, and the genes must be less than 1500bp long. Primers were developed using Primer3© (1998, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) as indicated in Table 2. Potential nonspecific binding of primers was examined in the MAP genome by comparing primers to the K-10 sequenced genome (GenBank Accession #AE016958.1). Briefly, two primers were designed upstream and downstream of the gene of interest. In addition, two primers were designed within the mariner transposon just past the inverted repeat region on either end. Four primer pairings per gene were run to amplify mutations in which the mariner transposon inserted in the correct orientation (Complement Primer Pairs: F + F = gene forward and mariner forward; R + R = gene reverse and mariner reverse) or if the mariner transposon inserted in an inverted direction (Figure 1; Alternative Primer Pairs: F + R = forward gene and mariner reverse; R + F = gene reverse and mariner forward).

Real-time PCR reactions were performed using a Bio-Rad C1000 Thermal Cycler (Hercules, CA, USA). Sso Fast Eva Green Supermix (Bio-Rad, Hercules, CA, USA) was used to prepare each reaction. All reactions were performed in a 40uL reaction mixture with 4 ng of DNA template. Forty cycles were performed per reaction. The software (Bio-Rad CFX Manager 2.0, version 2.0.885.0923, Bio-Rad, Hercules, CA, USA) determined the cycle threshold and calculated the Ct values for each reaction. To assess non-specific amplification, all primer pairs for each gene were evaluated by endpoint PCR; the presence of multiple amplicons on agarose gel were confirmed in the A1-157L compared to gDNA extracted from wt MAP. Due to the potential presence of template with the mariner transposon inserted at multiple positions in the same gene, the exact length of the amplicons was roughly unknown and sequencing of the resulting mix of amplicons was not possible. A gradient qPCR was performed for each primer pair on the inoculum DNA and compared to A1-157 wt genomic DNA to determine the idealz temperature for denaturation. Primer pairs that resulted in nonspecific amplification in the genomic DNA were rejected from further analysis.

Three independent amplifications were performed for each primer set for each gene. Samples with an undetermined Ct value were represented with a value of 40.0 to calculate the average for each triplicate. Each triplicate was evaluated for accuracy and triplicates greater than 1.0 Ct value apart were re-amplified. Precision melt analysis software (Bio-Rad CFX Manager 2.0, version 2.0.885.0923, Bio-Rad, Hercules, CA, USA) was used to evaluate the amplicon melting profiles for each triplicate. Outliers were identified and omitted from the average calculated. The delta delta CT method was calculated for each primer pair for each gene. T-tests were conducted using STATA (Stata/SE 13.1, College State, TX, USA) to compare change in

the relative quantification of each gene between the inoculum and the intestinal tissue and lymph nodes.

Targeted Next Generation Sequencing

Targeted Next Generation Sequencing was performed as described (Wang et al., 2014) on the DNA extracted from CFUs from all tissues collected at 2 mo PI; data from calf 5 were omitted due to a low bacterial recovery from its tissues (Table 1). Figure 2 diagrams the workflow for DNA preparation and sequencing synthesis. Briefly, DNA was partially digested, and ligated with asymmetric adapters. The DNA fragments with the Mariner transposon junction were amplified and Illumina sequencing indexed primers (Table 3) were added by nested PCR according to Griffin et al. (2011). Amplified fragments were gel purified and fragments between 250-400 base pairs were selected and sequenced with a generic Illumina primer (5′ACACTCTTTCCCTACACGACGCTCTTCCGATCT) using an Illumina HiSeq2000 system at the McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada). One hundred base pair reads were generated.

Sequencing Analysis and Bioinformatics

The transposon sequence (CGGGGACTTATCAGCCAACCTGT) was trimmed using cutadapt (Martin, 2011). Reads were aligned to the MAP K-10 reference genome (Li et al., 2005) using Bowtie2 alignment software (Langmead and Salzberg, 2012). SAMtools was used to convert Aligned Sequence Alignment/Map (SAM) files into binary BAM files (Li et al., 2009). Custom scripts in MATLAB® were used to analyze and map the reads to genomic TA sites. The

number of reads detected and strand orientation were determined for each TA insertion site. Each insertion site coordinate was mapped to a gene or region as annotated in RefSeq file NC_002944.2.ptt (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/Mycobacterium_avium_ paratuberculosis_K_10_uid57 699/NC_002944.gff). Read positions were visualized by either Integrative Genomics Viewer (http://www.broadinstitute.org/igv/) (Robinson et al., 2011, Thorvaldsdottir et al., 2013) or DNAPlotter (http://www.sanger.ac.uk/re-sources/ software/dnaplotter/) (Carver et al., 2009).

Essential Gene Analysis and Filtering

The following steps were taken to identify the essential genes from the bioinformatics results. All intergenic regions (IG) were removed from the data set. To eliminate the presence of insertion sites that were due to sequencing errors, TA dinucleotide sites with less than 100 reads in the inoculum were omitted from further analysis. The reads from each of the output pools were customized so that the number of identified mutants matched the colony counts obtained from each tissue. Therefore, TA sites with the lowest number of reads were omitted. Low numbers of reads were more abundant in samples with lower numbers of CFUs collected, and this removed background noise from the data. The remaining reads from each TA site for each tissue were reduced to binary data in which TA sites with ≥ 100 reads in the tissue received a score of 1 and TA sites with < 99 reads in the tissue received a score of 0. The binary data eliminates the effect that colony size might have on the output data which would have results in artificially higher reads.

All of the TA site for each gene were added together. The number of TA sites for each gene were counted. All of the genes were ranked to identify the top most essential genes: ascending order by most to least absences from the tissues and descending order by number of TA sites present. This revealed the top most depleted genes in all of the calves euthanized at 2 mo PI.

The percentage of reads from each TA site was calculated from the total number of reads recovered from the targeted NGS. This value was used to calculate the total number of CFUs of each mutant that were present in the inoculum generated from 100,000 CFUs prior to infecting the calves. The observed CFUs in all of the tissues was estimated using the reads from the targeted NGS for the inoculum and the total colony count for each sample. The expected number of CFUs was calculated for each of the absent mutants to determine the number of CFUs that would be required to be recovered if the mutant was nonessential for infection. The expected number of CFUs from each of the mutants was calculated using the percentage of the number of reads in the inoculum to the total number of TA sites in the MAP genome and multiplied by the total number of CFUs recovered from all of the tissues from each calf at 2 mo PI. The sum of the expected number of CFUs for each TA dinucleotide site was totaled for each gene for all calves. Lastly, the homologous gene sequences from *M. tuberculosis* (strain H37Rv) were used to determine the name and function of the genes absent from all tissues.

RESULTS

Fecal Shedding

MAP shedding in feces from both treatment groups (A1-157L and wt) was measured each month using qPCR for *IS900* and F57 (Table 4). All calves were shedding MAP bacteria at 1 and 2 mo PI. Three calves were still shedding at 4 mo PI and 5 calves were not shedding at all at 3 and 4 mo PI.

Calf Immune System Response to Infection

Calves euthanized 4 mo PI were evaluated for production of anti-MAP antibodies by measuring serum ELISA (Table 5). All calves were ELISA-negative for MAP-specific antibodies (S/P < 0.60). However, calf 14, inoculated with A1-157 wt, did have an increased sample to positive ratio (S/P = 0.45).

An IFN-γ response assay was performed for each calf at 2 and 3 mo PI with the exception of calves euthanized at 2 mo PI. For each sample, the results were validated by a significant response to phytohemagglutinin (PHA) and pokeweed mitogen (PWA). The average %IFN-γ production from each stimulant was calculated for all calves at 2 and 3 mo PI (Figure 3). %IFN-γ production to Johnin PPD was higher than the unstimulated negative control at both 2 and 3 mo PI ($p < 0.01$). %IFN-γ production after Avium PPD stimulation was higher at both 2 and 3 mo PI compared to the unstimulated negative control ($p < 0.01$). The %IFN-γ produced after stimulation to Bovine PPD did not differ from the unstimulated control at 2 mo PI ($p = 0.61$) but it was higher than the unstimulated sample at 3 mo PI ($p < 0.01$).

The %IFN-γ was also calculated separately for the positive control (A1-157 wt) and the treatment group (A1-157L) at 2 mo PI (Figure 4). Due to a small sample size of the positive control at the 3 mo time point, statistical analysis could not be performed. The %IFN-γ response to Johnin PPD calculated at 2 mo PI was higher than the unstimulated samples for both A1-157L ($p < 0.01$) and A1-157 wt ($p < 0.05$). The %IFN-γ produced after stimulation with Avium PPD was higher in the A1-157L treatment group ($p < 0.01$) but not for the A1-157 wt calves ($p = 0.19$). There was no difference in %IFN-γ production for either treatment group exposed to Bovine PPD when compared to the unstimulated sample ($p = 0.55$)

qPCR Relative Quantification

The proportion of specific mutants recovered from the intestinal tissues of the calves was compared to the proportion of the mutants in the inoculum. The selected genes had as few as 4 TA dinucleotide sites (gene 3406c) and as many as 29 TA dinucleotide sites (gene F57). The shortest gene chosen was 3406c (789 bp) and the longest gene was 4065c (1302 bp) based upon the sequenced genome of MAP strain K-10 (GenBank Accession #AE016958.1; Table 6).

Three anomalies observed were removed from all calculations because they resulted in significantly higher fold changes compared to the other data points and skewed the calculations: gene 4099c and 3406 in the ileum for Complement primer pairs F + F and gene 1466c in the ileum for R + F Alternative primer pairs. The reference gene, F57, had an average Ct value of 18.49 ± 0.41 in the inoculum and 18.98 ± 1.26 in all of the tissues evaluated suggesting that the presence of this native gene before and after treatment was very consistent. Fold changes were tallied for each gene for all primer pairs for decreases (fold change < 0.90), increases (fold change > 1.10), or no change (between 0.90 and 1.10) compared to the inoculum. When tallied,

all seven disrupted genes showed more decreases for all primer pairs in the tissues indicating an overall reduction in appearance of the mutant genes following treatment in the calves (Table 7). In addition, the number of colonies recovered from the different tissue samples, ranging from 929 CFUs to 27,313 CFUs, did not have an effect on the Ct values for the selected mutants recovered. In other words, tissues with lower numbers of CFUs recovered did not result in higher Ct values which would indicate that lower CFU recovery had less diversity or potential for those mutants to be recovered (Table 1).

Differences in fold changes of all of the selected mutants by tissue type was also considered. Due to a low sample size, the fold changes of all the disrupted genes were averaged for each of the primer pairs by tissue type (Table 8). The fold changes did not differ from 1.00 ($p = 0.16$) indicating that there was no effect by tissue type on the presence or absence of the mutants.

The average of the fold changes of the putative essential and nonessential genes was also calculated for all four pairs of primers, the complimentary primer pair, and the alternative primer pair (Table 9). No differences were observed when the fold changes of all four primer pairs were averaged by gene type ($p = 0.52$). For the complimentary primer pair, both putative essential and nonessential genes exhibited lower fold changes in the intestinal tissues (0.55; 0.88) and slightly higher fold changes in the lymph nodes (1.83; 1.06); however, these fold changes did not differ from each other ($p = 0.22$). The alternative primer pair did not exhibit a pattern, and, therefore, the fold changes between putative essential and nonessential genes did not differ ($p = 0.48$). Overall, this method demonstrates no differentiation between the selected putative essential and nonessential genes.

Targeted Next Generation Sequencing

Of the 52,834 TA dinucleotide sites in the MAP K-10 genome, 29,266 had been mariner transposon insertions in the pool of A1-157L mutants used as the inoculum (55% coverage). These insertions account for 3,729 gene disruptions of the 4,350 genes in the MAP genome (86%). There were 5,686 insertions in the IG regions which accounts for 19% of the TA dinucleotide insertions in the inoculum. To estimate the frequency in which each mutant appeared in the inoculum, the ratio of the reads from each TA site to the total number of reads recovered from the targeted NGS (~8 x 10^6 reads) was multipled by the total number of CFUs given to each calf (10^{11} CFUS). A histogram was used to represent the frequency in which the mutants appeared in the inoculum (Figure 5). The majority of the mutants in the inoculum were present in numbers between $10^{5.5}$ and 10^7 CFUs.

After filtering and removing IG regions, 12,998 TA dinucleotide insertion sites remained which disrupted 3,364 genes. There were 42 genes that had an average score of 0 which indicates they were the most depleted in the tissues (Table 12). Since not all genes in MAP have been identified, these MAP genes were compared to homologous genes in *M. tuberculosis* (strain H37Rv; GenBank Accession #NC_000962.3) to predict on their function in MAP survival. Seven of these top genes were hypothetical proteins with no reported function. There were two probable transmembrane proteins (MAP1531c and MAP3862c) and integral membrane proteins (probable, MA2562 and conserved, MAP0757). Six genes were associate with DNA transcription: DNA topoisomerase (MAP3244), an ATP-dependent DNA helicase (gene uvrD), and transcription regulators LysR (MAP1643), Gntr (MAP1267), TetR (MAP1267), and cprA.

Two of the top 42 most depleted genes from all of the calf tissues (MAP1505 and MAP1506) encoded for proteins belonging to the PPE (Proline-Proline-Glutamic acid) family

(Mi et al., 2016). Two genes which showed significant reduction in the calf tissue were an mce (mammalian cell entry) protein (MAP0765) and a possible mce-family lipoprotein (lprk). Fourteen genes among the top 42 genes were associated with metabolism or biosynthesis. Some examples include Isocitrate lyase (AceAb), which was ranked as the top gene from the calf tissues, is associated with replenishing intermediates for the Krebs cycle (Mukhopadhyay and Purwantini, 2000) which generates ATP through the oxidation of acetyl-CoA. In addition, a probable ATP-binding cassette transporter (MAP1531c) and an acetyl-CoA acetyltransferase enzyme (MAP2060c) were also top depleted genes. A serine peptidase (MAP1968c), mannitol-2 dehydrogenase (MAP0877c), a probable pyruvate carboxylase (pca), O-succinylhomoserine sulfhydrylase (metZ), and glucose-6-phosphate dehydrogenase (MAP3884) were also in the top 42 gene list. Catalyzing the transfer of electrons between biochemical reactions was also an essential component to MAP survival with four oxidoreductase enzymes on the list: glucose-methanol-choline oxidoreductase (MAP1385c), thiazolinyl imide reductase (MAP3744), cyclohexanone monooxygenase (MAP1463), and a possible oxidoreductase (MAP0081). In addition, there was one gene essential for DNA repair (uvrB), a lipoprotein (lpqZ), and a glycogen branching enzyme (glgB).

The sum of the reads recovered from the targeted NGS for each of the putative essential and nonessential genes that were selected for relative quantification using qPCR were reported in Table 10. The number of reads were compared to the Ct values obtained for each gene for each of the tissues in common. Numbers of reads from the targeted NGS did not consistently correlate with Ct values; in other words, high Ct values were not an indication of low numbers of reads and vice versa. In addition, there was no difference between the reads for the essential and

nonessential genes when compared to the inoculum in any of the four tissue types from this study ($p = 0.25$; Table 10).

The reads from the targeted NGS were calculated from the raw data from Wang et al. (2014). Table 11 summarizes the total reads for all disrupted TA sites for each gene from the targeted NGS for each of the putative essential and nonessential genes as well as the reference gene, F57, from each of the mice and the inoculum. Results from the targeted NGS on mice indicated that there was no difference between the number of reads for the essential and nonessential genes when compared to the inoculum in any of the mice ($p = 0.31$).

An enrichment analysis was performed on the top 600 essential genes for biological processes using Gene Ontology (Gene Ontology Consortium, www.geneontology.org). These genes included 591 in which all of their TA sites were missing from all of the tissues plus an additional 9 to round out the number. However, only the genes with a known *M. tuberculosis* homologue were included in the analysis which was 349 genes. The enrichment was calculated using the homologues discovered from *M. tuberculosis* (Table 13). Most of the genes were associated with mycobacterial growth (n = 110). Oxidation-reduction processes (n = 75) are also extremely important for MAP survival in the calves. Responding to stimulus (n = 77), symbiosis and parasitism (n = 43), pathogenesis (n = 26), and DNA repair (n = 18) complete the top biological processes associates with MAP survival in dairy calves.

The 42 top most depleted genes were compared to the published list from Wang et al. (2014) and presented in Table 14. The MAP K-10 strain used in the mice shows no overlap with any of the top *in vivo* genes presented in the current study from MAP strain A1-157 in cattle. Although there were no genes in common between the two studies, some of the genes shared similar functions. For example, several oxidation-reduction enzymes were essential *in vivo* in

mice such as MAP2385c, MAP2008, and MAP1082c. A PPE family protein (MAP3420c), which is associated with virulence in *M. tuberculosis* was absent from all mice; two PPE proteins were absent in the dairy cattle. Overall there is little similarity between the essential *in vivo* genes determined from both studies.

DISCUSSION

Inoculum

The purpose of this study was to evaluate genes that are critical for survival of MAP in dairy calves. A large input pool of mutants was generated which, following infection, was evaluated using sequencing technology. All individual TA dinucleotide sites in individual mutants that had accepted the transposon insertion were sequenced and analyzed on the whole genome level. In a previous study using the same approach to study MAP survival in a mouse model, the inoculum produced by Wang et al. (2014) successfully interrupted 24% of the TA dinucleotide sites and 56% of the genes in the MAP K-10 genome. The A1-157 library used in this study and developed using the same technique (Tn-seq) had significantly more coverage. This is likely due to the combination of three independent libraries, a suggestion provided by Joyce Wang and Marcel Behr (personal communication). The ability to represent more TA dinucleotide sites provided the opportunity to analyze more potential essential genes *in vivo* and increased our confidence of identifying virulence genes because multiple TA sites per gene could be analyzed in parallel. In addition, each calf received an inoculum in which the majority of mutants had at least 10^6 CFUs present which is considered the minimum infectious dose for MAP (Sweeney et al., 2006). This means that most of the mutants were administered at an infectious level, and their absence from the cultured tissues was not a reflection of too few mutants available for infection.

Inoculating the calves in our study with an extremely high dose (10^{11} CFUs) ensured adequate recovery for downstream sequencing. A minimum dose of 10^6 CFUs of MAP can reliably produce an infection in newborn calves 3 wk after inoculation (Sweeney et al., 2006),

however, we needed to recover at least 100,000 CFUs for targeted NGS (Murry et al., 2008). The mouse infection model is more artificial in that the mice are not orally inoculated but rather intraperitoneally injected which allowed for adequate MAP recovery (Wang et al., 2014); we therefore needed to consider a higher dose than that given to the mice (10^8 CFUs). A previous trial inoculated goats with 10^9 CFUs of MAP strain K10 2 mo prior to necropsy. Numbers of CFUs from 1.5 g of tissue ranged from 2 to 285 in the intestinal tract and associated lymph nodes (Park et al., 2011). However, another caprine trial inoculated 1.44 x 10^9 CFUs over 2 d using a K10-like isolate recovered much higher CFUs per g of tissue (53-1440 CFUs/g). In addition, there was significant variation between the individual goats (Hines et al., 2014).

Another study demonstrated that the number of CFUs recovered from a specific mesenteric lymph node directly correlate with the corresponding intestinal segment in which it drains, though lower numbers were recovered (Sweeney et al., 2006). Therefore, it was imperative that we culture a greater amount of tissue from both the intestines and lymph nodes in order to obtain enough CFUs for the targeted NGS. With so much variation noted in previous animals, we chose to inoculate with a dose 100 times greater than the previous studies to increase the amount of bacteria that can infect the gastrointestinal tract. In the current study the required amount of bacteria isolated from the tissues was only obtained by combining all of the CFUs obtained from the calves euthanized at 2 mo PI. It is likely that culturing more tissue would have resulted in obtaining 100,000 CFUs for some of the individual calves CFUs though it is unlikely to have achieved this for all calves.

A minimum set of genes is necessary for all bacterial life; these genes are typically referred to as housekeeping genes (Gil et al., 2004). Without these basic types of genes, an organism would be unable to survive. By utilizing the sequenced genome of *Mycoplasma*

genitalium, the cellular life form with the smallest known number of genes (468 genes, Fraser et al., 1995), and comparing it to *Haemophilus influenzae*, at the time of publication, the other completely sequenced small bacterial genome (1703 genes, Mushegian and Koonin, 1996). The researchers reasoned that by examining the two smallest known genomes capable of cellular function, they could determine the set of minimally required genes that evolved for all bacteria species. They identified 256 genes identified that were responsible for cellular replication, recombination and repair, DNA transcription, RNA translation, metabolism, energy, uptake of inorganic ions, exopolysaccharides, and chaperone functions (Mushegian and Koonin, 1996).

In addition to the minimum set of genes required for most bacterial survival, MAP must uptake iron; mutations to its iron-chelating pathway could have deleterious effects rendering it unable to survive *in vitro*. Unlike other microbes that produce their own iron-chelating siderophores for iron uptake from their surroundings, MAP requires supplementation of mycobactin, an iron-binding siderophore, isolated from *Mycobacterium phlei,* to grow in the laboratory (Francis et al., 1953). While iron acquisition has been extensively studied in *M. tuberculosis,* and 2 gene clusters for iron-binding have been identified (*mtb-1* and *mbt-2*; De Voss et al., 1999, Krithika et al., 2006), little is known about iron chelation in MAP. Numerous deletions occur in the mycobactin biosynthesis pathway in the MAP genome suggesting an alternative pathway is required (Wang et al., 2015). It has been speculated that a specific genomic island or large sequence polymorphism called LSP^P15 may encode for several metal uptake systems (Wang et al., 2015). Though iron chelation in MAP is still being investigated, it is essential for MAP survival, and mutations in this pathway would have deleterious effects on MAP's long-term survival and replication.

Therefore, representing 100% of the MAP genome with mutant strains is unlikely since some mutations will have deleterious effects on *in vitro* survival. To remove potential mutants that would not be capable of growing *in vitro* the initial steps in developing and selecting the mutants involves growth on solid media. Our inoculum successfully represented 55% of the TA dinucleotide sites and 86% of the genes in the A1-157 genome. Assessing 3,729 genes for virulence provides many potential targets for attenuation to develop a vaccine strain. In addition, the 14% of genes not represented could be required for *in vitro* survival. However, this would suggest that every gene in the MAP genome was mutated which is unlikely to occur. Therefore, these genes would need to be investigated further. This study has provided an excellent database for investigating genes that are essential to MAP for replication, homeostasis and essential metabolic functions.

Culturing MAP on Solid Media

Differences in host genetics and how the individual calves responded to MAP infection may account for individual variations in MAP infection and immune responses. Jejunal LN from red deer that were relatively resistant or susceptible to MAP were sequenced for differences in gene expression (Mackintosh et al., 2016). The 9 genes upregulated at 4 weeks PI in resistant animals were all associated with host defense involving Type I IFN-γ stimulated cells while the susceptible deer had an immune response predominantly related to inflammation, an adaptive immune response, mitochondrial function, and apoptosis. In addition, a review article highlighted the need for further information in production animal susceptibility and resistance to MAP infection and suggested that selective breeding may assist in MAP resistance (Vir Singh et al., 2013).

There was considerable variation in the number of CFUs cultured from each of the calves (Table 4). All calves received the same amount of CFUs from the same pool of either A1-157 wt or the A1-157L of mutants. Some calves exhibited significant variation in CFUs between each of the four tissues. For example, calf 9 had very high numbers of CFUs recovered from both the IL and JEJ but much less was cultured from the lymph nodes (Table 4). Calf 14, one of the positive control calves, had such high numbers of MAP from 3 out of 4 of the tissues cultured that they were too numerous to count. This was unusual compared to the other 15 calves. In addition, it is important to note that higher numbers of colonies were collected from fresh tissues compared to frozen tissues. Fresh tissues were cultured the day of collection. To obtain higher numbers of CFUs from the tissues and also to re-culture some of the tissues that resulted in too much contamination from other bacteria, frozen tissues were cultured at a later date. However, many of the tissues yielded very low results and some did not yield any CFUs at all. No reports on studies could be found on the viability of freezing MAP in tissues. However, previous research on frozen MAP-contaminated fecal samples suggest significant losses between 0 and 3 wk of freezing (Richards and Thoen, 1977). However, a later study suggested that not only were losses from long term frozen storage not significant, but multiple re-freeze thaw cycles did not significantly impact MAP viability (Raizman et al., 2011). Therefore, it is currently inconclusive whether MAP viability in intestinal tissues or lymph nodes is affected by long term storage at -80°C.

Calf Immune System Response to Infection

None of the calves exhibited an antibody response to MAP infection (Table 5).

However, calf 14, from which the most CFUs were cultured, had with 0.45 the highest S/P ratio. A previous long-term infection trial indicated that calves receiving a high dose of MAP (10^{10} CFUs) are more likely to exhibit a humoral immune response to infection (Mortier et al., 2014a). In addition, out of the 5 calves inoculated at 2 wk of age with this high dose, 2 were ELISA-positive at 4 mo PI. Two of the remaining calves did not become ELISA-positive until after 7 mo PI. This suggests that while it is possible for the calves to develop an immune response as early as 4 mo, there is considerable individual variation. Research on early antibody detection to MAP in dairy cattle is limited since cattle that appear healthy do not usually undergo additional tests. A few exceptions evaluated early antibody response to MAP infection in dairy cattle (Koets et al., 2001, Eda et al., 2006). Waters et al. (2003) and demonstrated that calves inoculated weekly with 10^6 CFUs of MAP for 3 wk elicited a lipoarabinomannan-based antibody response 134 d PI. However, the IDEXX Paratuberculosis AB Test could not detect a serum antibody response in any of the calves for the entire 10-mo trial. The researchers suggested that the IDEXX test could have been designed to detect antigen expressed later in the disease progression. While it was previously recommended to test for humoral immunity after 2 yr of age (Lepper et al., 1989), both Waters et al. (2003) and Mortier et al. (2014a) have demonstrated the capability of antibody detection earlier in MAP infection.

IFN-γ production was measured to demonstrate that the treatment calves were successfully infected with the A1-157L. Samples from all calves responded to the PHA or PWA antigens which served as positive controls. The Johnin PPD, derived from MAP, induced the greatest IFN-γ expression though, at 3 mo PI, it did not differ from IFN-γ produced in response to Avium PPD (Figure 3). The similarity in IFN-γ production is likely due to the fact that Avium PPD is derived from *Mycobacterium avium* subsp. *avium* which belongs to the same species as

MAP (Turenne et al., 2008). Bovine PPD elicited the least amount of IFN-γ production which was to be expected since it is derived from *M. bovis* (Jungersen et al., 2002) which is not as similar to MAP. IFN-γ is crucial in the immune response to intracellular pathogens, especially mycobacterial species. The immunostimulatory and immunomodulatory effects stemming from IFN-γ production activate proinflammatory cytokines which contribute to macrophage activation allowing the host to clear the infection (Schoenborn and Wilson, 2007). The production of IFN-γ elicited by the calves to Johnin and Avium PPD indicate their immune systems are responding to MAP thus all calves were successfully inoculated.

Relative qPCR Quantification Method

The qPCR relative quantification method was explored as a more targeted and less expensive tool, compared to transposon-sequencing, for evaluating the quality of the inoculum and determining if essential genes for the survival of MAP in mice could be confirmed as such in a dairy calf infection model. The quality of the inoculum can be measured in terms of the number of genes with mutations, the number of TA insertion sites, as well as the relative quantity of each mutant. Although qPCR cannot measure the number of insertion sites in each gene, it can be used to reveal the relative quantity of each mutant gene in a given sample.

In this experiment the Ct value can be interpreted as an estimation of individual bacteria with mutations in a specific gene in a particular sample. Relative quantification relates the Ct values of the specific target following a treatment to the untreated control (Livak and Schmittgen, 2001) which in this case is the inoculum prior to infection. F57, which served as a reference gene, was selected because of its specificity to MAP and the presence of only a single copy in the MAP genome (Coetsier et al., 2000). In this experiment, the primers for F57 were

specific for the intact gene and not for F57 mutants. The reference gene, F57, is meant to quantify the total number of copies of the MAP genome in contrast to the primer pairs, designed to include the transposon, which measured the number of mutants present in each targeted gene. The Ct values from the inoculum and the average of the Ct values from the tissues suggest that F57 was consistently present despite incubation in the calf tissues and served as an excellent reference for the delta delta Ct method. The genes were selected from a previous publication by Wang et al. (2014), which reported putative essential genes in MAP. In addition to validating relative quantification as a method for evaluating the inoculum this method was also proposed as a way to validate whether the essential genes suggested by Wang et al. (2014) were also essential in dairy calves.

More TA sites were disrupted in the three independent libraries produced for this trial compared to Wang et al. (2014) for all but two of the selected genes which had the same number of disrupted TA sites (Table 6). Calculations made from the raw data obtained from Wang et al. (2014) show no difference between the number of reads in the inoculum compared to the mice spleens from the targeted WGS (Table 11). Therefore, the calculated fold changes from the relative qPCR quantification do not confirm the putative essential genes as reported by Wang et al. (2014).

The results obtained with this qPCR method are inconclusive for the selected genes and their relevance for MAP survival in dairy calves. The relative quantification of the inoculum and the CFUs obtained from culturing was performed prior to the targeted NGS. The reads for each of the selected genes in the outputs was compared to the Ct values to determine if there were any similarities. It is interesting that some mutant genes were present in some tissues and, though inconsistent, disappeared in others. For example the relative quantification for gene 1082c, a

putative essential gene, indicated there was sporadic recovery in the tissues. The results from targeted NGS indicate that some TA sites from this gene also appear sporadically in the tissues (Table 10). It is interesting that the tissues with lower numbers of reads from the targeted NGS (11 IL, 12 IL, 4 JEJ, and 4 JLN) appear to correlate with higher Ct values. However, this did not correlate for all the tissues from 1082c; 2 IL and 9 ILN exhibited high numbers of reads but did not have correlating low Ct values.

It is possible that transposon insertions in certain TA sites were preferentially amplified by qPCR due to the size of the respective amplicon. A transposon mutant with an insertion in the beginning of the gene would be a smaller amplicon than an insertion at the end of the same gene based upon the primers designed. If mutants were recovered in a specific tissue with a mutation at the end of the gene, this may result in a higher Ct value but still provide high numbers of reads in the targeted NGS which is more specific for each TA site. This may explain the variable Ct values which could contribute to the inconclusive fold change results.

Another example of targeted NGS correlating with relative quantification is the putative essential gene 3741 which had consistently low fold changes except for some variability in the JLN. The targeted NGS indicates that most of the TA sites from gene 3741 were absent from the tissues with the exception of the JLN (Table 10). While the relative qPCR results were not definitive, the most variation in fold changes occurred in the JLN, the tissue type which resulted in the most reads from targeted NGS. However, it is important to note that this does not occur for all of the genes. For example, targeted NGS recovered relatively high numbers of the 2 TA sites in putative nonessential gene 3406c while the calculations from relative qPCR exhibit very low fold changes in most of the tissues. The reason for the discrepancies between the 2 methods remains unknown. However, the inefficiencies with the experiment could be due to the

limitations observed with this method which prevent it from being advantageous for this research project. These limitations will be discussed in detail below.

Not enough was known about the efficiency of each primer pair to know if they were functioning optimally for the relative quantification. While each primer pair was tested for amplification of only the gene of interest and discarded if nonspecific binding occurred, there are still many variables that could affect the efficiency of the reactions. Each gene varied in length the shortest being 789 bp and the longest was 1302 bp. With the addition of the mariner transposon, each mutated gene was lengthened by 2149 bp. While the primers for the mariner transposon were designed approximately 200 bp inside of the sequence past the inverted repeat region, this still added considerable length to some of the amplicons (Table 6) depending on where the disruptions are located in the gene. Amplification of variable lengths could account for the sporadic Ct values observed in the 4 sets of primers used for each gene. It is possible that the inconsistent lengths of the amplicons were reflected in the Ct values calculated in the different tissues as well.

Shorter amplicons are more likely to be denatured during the denaturation step in the qPCR reaction allowing primers to more efficiently bind to their complimentary regions (Jozefczuk and Adjaye, 2011). Because of this the ideal length of an amplicon for qPCR is 150bp though amplicons up to 300 bp can also be tolerated (Saunders and Lee, 2013). The Taq polymerase is also more effective on shorter sequences since it has an extension rate of 30-70 bases per sec; typical extension times occur only a few seconds (Bio-Rad, Hercules, CA, USA). The qPCR protocol for this project had an extension time of 30 sec, a standard extension time used in many protocols, which may be most efficient for amplicons less than 420bp. Also, shorter sequences are more likely to be amplified compared to longer ones. Therefore, if the

mariner transposon were inserted towards the end of the gene, compared to an insertion site that was closer to the forward primer's complimentary region, that mutant will be less likely to be amplified resulting in a skewed Ct value.

For the purposes of mutating many genes the key feature of the mariner transposon is that it can insert itself after any TA site. This becomes a caveat for the qPCR quantification project since the 7 selected genes had as few as 4 TA sites and as many as 19. If the most efficient amplicon is only 150 bp in length, then primers would need to be designed for each possible TA site for each gene to only amplify a very short sequence. It would be very impractical to design this many primers for each gene to account for all of the possible outcomes.

Performing statistical analyses on this data set proved difficult. Due to a small sample size for each tissue, the data set lacked the power for effective statistical testing. Attempts were made to group the intestinal tissue and lymph nodes, however, the sample sizes were still too small. In addition, the different primer pairs could also not be grouped together since they represented two different orientations for the mariner transposon. When examining the averages for the primer pairs, we were able to group together the complimentary pairs of primers and the alternative primer pairs since they amplified the same mariner transposon orientation. Treating each gene and each primer pair independently would have created a multiple testing problem resulting in the possible outcome that incorrectly rejects the null hypothesis.

This project was able to identify the shortcomings and caveats for qPCR-relative quantification. While this was not the most conducive method for this research project, it did provide early insight into the diversity of the inoculum prior to receiving the data from the targeted NGS. Learning about the pitfalls of this project provided the opportunity to explore all

facets of troubleshooting qPCR and use foresight when investigating future projects and looking for limitations and impediments.

Targeted Next Generation Sequencing

Following incubation in MAP's natural host, targeted NGS of a pool of mutant strains of MAP was used to examine potential virulence genes essential for infecting the tissues of dairy cattle. This method uses massive parallel sequencing of short reads of millions of DNA strands which provided a detailed picture of the mutants present in the initial inoculum and compared it to the mutants that were recovered from each of the tissues from 5 infected calves at 2 mo PI. To eliminate the presence of insertion sites that were due to errors from the sequencing, TA dinucleotide sites with less than 100 reads in the inoculum were removed. This eliminated background noise from the sequencing to account for stochastic effects and eliminate potential false positives. In addition, analyzing the data at the whole gene level and not individual TA sites provides further protection from reporting false positives. This resulted in many genes with a high probability of being significant for the survival of MAP in dairy cattle. Below some of the genes are described in detail and speculation into their potential role for MAP virulence.

Bacteria with mutations in genes associated with metabolism and biosynthesis were significantly reduced in the calf tissues and may possibly serve as vaccine candidates. The most essential gene absent from the calf tissues was aceAb or isocitrate lyase (ICL). Adenosine triphosphate (ATP) is essential intracellular chemical energy. Isocitrate lyase (AceAb), is associated with replenishing intermediates for the Krebs cycle (Mukhopadhyay and Purwantini, 2000) which generates ATP through the oxidation of acetyl-CoA. It is also very important for resistance to antibacterial drugs used for treating *M. tuberculosis.* Three anti-tuberculosis drugs

(isoniazid, rifampicin, and streptomycin) activate ICLs in *M. tuberculosis*. ICL-deficient *M. tuberculosis* is much more susceptible to these 3 antibiotics suggesting that ICL plays a role in antibiotic resistance (Nandakumar et al., 2014). It has also been suggested that for *M. tuberculosis* to maintain a quiescent or non-replicating state that ICL must be upregulated (Wayne and Lin, 1982). MAP remains in its primary host for 3 to 5 yr before signs of clinical disease manifest. While the bacteria continue to replicate and shed in the feces, they maintain a steady state within the host for a considerable amount of time. It is possible that ICL maintains a similar mechanism for MAP to persist in the intestinal tract of dairy cattle, however, there is currently no published research investigating its role in MAP survival.

Pyruvate carboxylase is also an important enzyme for producing intermediates of the TCA cycle. Pyruvate carboxylase is highly homologous in *M. smegmatis* and *M. tuberculosis*. Similar to ICL it is suggested that the anaplerotic or intermediate reactions produced by pyruvate carboxylase play an important role in a persistent, non-replicating stage of *M. tuberculosis* (Mukhopadhyay and Purwantini, 2000). This suggests that further investigation into the enzymes required for the production of TCA cycle intermediates could elucidate their role in MAP survival. It is possible that enzymes responsible for anaplerotic reactions may serve as future targets for vaccine development.

An mce protein (MAP0765) and a possible mce-family lipoprotein (lprk) were significantly reduced from the cultured calf tissues. The mammalian cell entry (mce) proteins are essential for the persistence and virulence of *M. tuberculosis* in the host (Forrellad et al., 2013). While the genes exist in several different bacterial species, only in mycobacteria do the *mce* genes act as an operon. The MAP genome contains eight *mce* operons which encode 74 different mce proteins (Timms et al., 2015).

The 2 PPE family proteins absent from the calf tissues after 2 mo PI are members of the ESX-5 system which is associated with virulence in *M. tuberculosis* (Mi et al., 2016). ESX-5 is the only Type VII secretion system present in slow-growing mycobacterial species (Abdallah et al., 2009) and have been suggested to be associated with pathogenesis and virulence in *M. tuberculosis* (Gey van Pittius et al., 2006). ESX-5 is involved in the secretion of many proteins which contain PE (Proline-Glutamic acid) and PPE sequences. *Mycobacterium marinum* ESX-5 mutants are unable to modulate the cytokine response in human macrophages (Abdallah et al., 2008). The PPE proteins are associated with virulence in *M. tuberculosis* (Ramakrishnan et al., 2000). Though not the same as the 2 PPE proteins absent for the calf tissues, expression of PPE25 and PPE26 in non-pathogenic *M. smegmatis* resulted in enhanced survival in macrophages and persistence in mouse tissues (Mi et al., 2016).

There are 36 PPE orthologues between the H37Rv strain of *M. tuberculosis* and the MAP K-10 genome, however, there are no studies reported that have examined their role in MAP (Gey van Pittius et al., 2006). MAP1506 was previously detected by mass spectrometry, and confirmed to be expressed on the cell surface of MAP (Newton et al., 2009). While very little is known about the 2 PPE proteins absent in the current trial, they may play an important role in pathogenesis. Reducing or eliminating their ability to be secreted by MAP may have significantly hindered its survival in the dairy calves.

Several DNA transcription regulators were also responsible for MAP survival in the primary host. Transcription regulators have been the target of anti-tuberculosis treatments (Ravishankar et al., 2015). Topoisomerase is responsible for relaxing the supercoils of DNA to allow for transcription to occur (Lodish H, 2000). *Mycobacterium tuberculosis* contains only a single copy of topA, topoisomerase I. Downregulation of topA results in decreased viability.

Furthermore, a topA knockout was cleared from the lungs of mice 2 mo after inoculation (Ravishankar et al., 2015). A follow-up study showed that small molecule inhibitors of topoisomerase I had antibacterial effects on both *M. smegmatis* and *M. tuberculosis* (Sandhaus et al., 2016). The disrupted transcription regulators in the A1-157L did not inhibit growth in an enriched environment (liquid and solid media) as evident by its present in the inoculum, however, the stressful conditions of the intestinal epithelial environment inhibited replication and prevented survival of these mutants.

LysR-type transcriptional regulators are thought to be the largest family of transcription regulators; their functions have been identified in very diverse bacteria and eukaryotes. They regulate many cellular activities such as responding to oxidative stress, cell division, protein secretion, and even virulence (Maddocks and Oyston, 2008). Protection against oxidative stress or reactive oxygen species is crucial for survival of *M. tuberculosis* (Manca et al., 1999) and possibly MAP (Liu et al., 2001). Interestingly, in *M. tuberculosis* a LysR-type regulator called OxyS is an oxidative stress response regulator. It directly binds with the promotor region for the *katG* gene which encodes a catalase-peroxidase to protect the bacteria from ROIs (Li and He, 2012). This LysR-type regulator is directly associated with *M. tuberculosis* virulence. MAP1643 is also a LysR transcription regulator. Further research to elucidate the role MAP1643 plays in MAP survival may reveal a gene which is also virulent.

Previous studies have evaluated many different candidate genes to knockout for potential vaccine strains. The data from our study can be used to impartially evaluate how well these knockouts can survive in a calf model. The *leuD* mutant (MAP3025c) evaluated by Faisal et al. (2013) induced a prolonged IFN-γ response in goats but did not prevent infection or fecal shedding. This mutation had less than 10 reads represented in our inoculum which resulted in no

reads in the output pools; there were no copies present in the Wang et al. (2014) inoculum. Therefore, it is impossible to draw conclusions from this data on the probability of *leuD* as a vaccine strain candidate. A knockout of the *relA* gene (MAP0093) was investigated in goats and cleared from the tissue. In addition, a minimal number of CFUs from the challenge strain K-10 were recovered (Park et al., 2011). The targeted NGS data showed that 5 out of 12 TA sites were represented by a total of 372 copies in the inoculum. This gene was ranked 125th. Based on this gene's ranking in our data and the previous research performed by Park et al. (2011), it is possible that a *relA* knockout is a good vaccine candidate, however, further investigation such as a challenge trial in dairy cattle still needs to be performed.

Compared to the genes reported by Wang et al. (2014), there were no essential virulent genes in MAP strain A1-157 and K-10 in common between the mouse and cattle model. This could be due to different evaluation methods. Therefore, the raw data from the K-10 experiment were evaluated by a similar method to the data obtained from the A1-157L. The TA sites were compiled by gene and filtered for IG regions and genes with less than 100 reads were removed from further evaluation. Using this method, no essential genes were recovered from the K-10 data set. Wang et al. (2014) did not evaluate the genes by compiling all of the TA sites together; instead, the genes were reported by each TA site, and they included all TA sites with greater than 1 log (10 reads) in the inoculum. Evaluating the data by TA sites only may have resulted in false-positive genes being reported as possible essential genes. Also, by including such low numbers of reads in their inoculum calculations they may have included erroneous data in the targeted NGS sequencing process (Wang et al., 2014). Therefore, we cannot make any conclusions regarding essential virulent MAP genes in both mice and dairy calf models.

However, the current trial was not without shortcomings of its own. To ensure adequate representation of the whole MAP genome, 100,000 CFUs needed to be represented in the inoculum and recovered from each tissue (Murry et al., 2008, Wang et al., 2014). While enough CFUs were input into the inoculum, not enough CFUs were recovered from each animal let alone each tissue. To obtain enough bacteria from each tissue type, a large amount of tissue had to be cultured. Devising an effective protocol for decontaminating the intestinal tissue of endogenous bacteria and extracting MAP from within the macrophages of the tissue was difficult to accomplish without experiencing some loss of bacteria. After culturing fresh tissue and acquiring less than 100,000 CFUs, additional tissue was cultured. However, the tissues that were frozen at -80°C resulted in a considerably lower yield. In order to evaluate the mutants, we had to combine all tissues and all calves at the 2-mo time point. Numbers of CFUs recovered from the calves that incubated for 4 mo PI were too low to combine together, therefore, they were omitted from Tn-seq analysis.

This type of experiment also features a caveat that may influence the mutants that do survive. Because of the amount of bacteria each calf was inoculated with and the number of different types of mutants, it is likely that some mutants were rescued by other mutants. For example, multiple mutants can be phagocytosed by one macrophage (Bannantine and Stabel, 2002, Keown et al., 2012). It is possible that a fatal mutant is capable of being rescued by another mutant that can survive the harsh environment within the macrophage and prevent phagocytosis. These rescue situations can occur at any major barrier imposed by the host such as infiltration of the intestinal epithelium; a MAP mutant unable to be opsonized could migrate through the gap junctions produced by other mutants (Bannantine and Bermudez, 2013). It is unknown how often these rescue situations occur but it is possible to alter the ratio of mutants

that are recovered which could lead to false positives. This experiment makes assumptions that mutant rescue may occur but occurs at such a low rate that it does not interfere with the recovery of mutant MAP. To improve upon this type of experiment a greater amount of fresh tissue should be utilized to harvest bacteria.

FUTURE DIRECTIONS

Effect of -80°C Storage. Freezing samples at -80°C is necessary for MAP processing at a later date. Some processes take a long time and, in many cases, experimentation cannot be performed immediately on fresh tissue due to the length of an arduous necropsy or scheduling conflicts. This project demonstrated that freezing tissue at -80°C resulted in significant loss of bacteria when compared to culturing fresh tissue samples. There are currently no reports of studies that evaluate the viability of MAP in tissues after freezing. Various tissues should be frozen at both -80°C and -20°C. Thaw and re-freeze cycles that may affect MAP viability can also be investigated. It is also important to test the different states in which the tissue is frozen: a solid piece, just the scraped mucosa, or as a frozen homogenate. Knowing the best way of storing MAP in tissue and fecal samples at various temperatures will ensure optimization of future experiments.

Host Genetics. Another interesting topic that requires additional research is host genetics in relation to MAP immune response and resistance to clinical disease. Why do some cows never develop clinical symptoms of JD but continue to shed MAP? On the extremes, very few colonies were cultured from all 4 tissues from calf 7 while the tissues from calf 14 were harboring so many colonies they were too numerous to count. While one calf received the mutants and the other received the wt strain, it begs the question why such extremes in infection were observed. Understanding host genetics may provide insight into selecting future breeding stock for farms (i.e. choose the cows that are less likely to have high MAP infections). Genomic sequencing on host genes associated with immune response, intracellular bacteria response, or other important biological factors could shed light on genetic differences in cattle that predispose them for MAP infection.

Evaluate Vaccine Candidates. The results from the research trial demonstrate that many genes are essential for MAP survival in dairy cattle. However, the top most depleted mutants represent genes that could be potential vaccine candidates for the prevention of JD in dairy cattle. Some of the top 42 genes with the lowest scores and greatest number of TA sites represented in the inoculum have the most potential for success. These genes should be further investigated by creating knockouts and testing their ability to survive in bovine peripheral blood mononuclear cells (PBMCs).

Shenkerman et al. (2014) have developed a novel and advanced allelic exchange protocol to knockout (KO) genes in mycobacterial species. This protocol improves current gene KO systems by combining high homolog recombination rates with a more efficient screening method to recover a greater number of mutants making this an efficient method for evaluating the top most depleted mutants from our research trial. The first step requires the recombineering proteins—recombination proteins used for genetic engineering—gp60 and gp61 from mycobacterial bacteriophage Che9c which catalyze the insertion of a linear piece of DNA (Che9c 60-61, van Kessel and Hatfull, 2007). Che9c 60-61 offers a more efficient method for homologous recombination and results in less non-specific recombination in both *M. smegmatis* and *M. tuberculosis* compared to other allelic exchange systems. Plasmid YS1 is a kanamycin resistant, temperature-sensitive mycobacterial suicide shuttle vector designed to express Che9c 60-61 under an acetamidase promotor. Adding acetamide to the growth media at a permissive temperature (34°C) can induce expression of Che9c 60-61 in mycobacteria. Flanking regions homologous to the target sequence direct the linear DNA containing a cassette with GFP and hygromycin resistance flanked by *loxP* into the host cell. Using both GFP and hygromycin offers

an easy method to identify mutants. Plasmid PYS2 was designed as a simple cloning vector for the homologous flanking regions around the *loxP-gfp-hyg-loxP* cassette.

Once the recombination event occurs, the Che9c 60-61 genes should be eliminated. Plating the cells on sucrose-containing media and incubating at a restrictive temperature (42°C) selects for both recombinant host cells and cells cured of plasmid YS1. GFP positive and hygromycin-resistant colonies are selected. Finally, the cassette containing the GFP and hygromycin resistance is excised from the chromosome resulting in an unmarked deleted mutation. Removal of the *gfp-hyg* cassette is accomplished with plasmid pML14 which contains a temperature-sensitive mycobacterial origin of replication and the *cre* gene (Ofer et al., 2012). The Cre enzyme recombines *loxP* at a permissive temperature of 34°C resulting in the excision of the *gfp-hyg* cassette. Growing colonies as 42°C on solid media lacking antibiotics facilitates the curing of plasmid pML14 resulting in the recovery of KO mycobacteria.

Once the KOs have been created, it is important to characterize the role of the genes and their ability to survive in the host. Many *in vitro* methods can be used to determine the role virulence genes have on MAP survival. Replication and survival of the KO MAP strains in bovine macrophages can be evaluated as previously described by Lamont et al. (2014). Microscopic magnification will be used to determine the percentage of macrophages containing mutant MAP and also to count the number of bacteria per macrophage over various time points post-infection.

ICL, the top most depleted gene from this trial, may be an excellent target for MAP attenuation. However, it's role in MAP survival is not yet known. It is possible that, similar to *M. tuberculosis,* ICL helps to maintain a quiescent steady state in MAP within the host. In addition, while the role of some PE and PPE proteins have been investigated in other mycobacterial

species such as *M. tuberculosis* (Gey van Pittius et al., 2006, Mi et al., 2016*)* they have not been investigated in MAP. While MAP1506 is found on the cell surface (Newton et al., 2009), it is unknown if there are other surface PPE antigens that could be potential targets for attenuation, if they are important for pathogenesis like they are in other mycobacterial species (Abdallah et al., 2009). Investigating both ICL and PPE protein activity in bovine PBMCs infected with MAP may elucidate its role in survival. Another option for investigating ICL activity(Newton et al., 2009) is by utilizing a ligated ileal loop model. Lengths of distal jejunum and ileum are ligated leaving an inter-loop space for short intervals (Khare et al., 2009), or the loop can be anastomosed or connected with a blood vessel for a longer infection period (Charavaryamath et al., 2013) before being inoculated with MAP bacteria. At specific intervals, loops can be excised, the bacteria collected, and RNA expression for ICL evaluated. Knowing more information about how MAP maintains a steady state during long term infection may provide some insight into vaccine development.

An ideal vaccine would be completely cleared from the host. However, complete clearance may not be possible. MAP is very hardy and its capacity to survive harsh environmental conditions and sustain survival for a considerable length of time means that reinfection is more likely to occur on farms with positive environmental samples. Its ability to maintain a quiescent steady state, survive in macrophages, and camouflage itself from the host immune system makes it unlikely that that the host immune system would be able to provide sterile immunity from MAP. Therefore, aiming for an effective vaccine that induces a strong immune response to eliminate fecal shedding and also prevent the development of clinical symptoms would be very beneficial to the dairy industry. If the vaccine can prevent on-farm

financial losses due to reduced milk production and increased culling, then it will be a worthwhile investment for researchers and farmers.

CONCLUSIONS

Overall, this experiment was very successful. A high number of mutants were harvested from the tissues and successfully compared to the input pool in the inoculum filling a knowledge gap regarding essential genes for *in vivo* survival in the primary host. A protocol was developed to successfully isolate MAP bacteria from a large amount of tissue which has also not been reported before. While the relative quantification qPCR project was unsuccessful, targeted WGS provided a multifaceted technique to evaluate millions of reads and reveal the relative frequency in which numbers of specific mutants occurred in tissues compared to the inoculum.

Table 1. CFUs collected from all calf tissues.

* = tissues used for Relative Quantification. ** = mo post inoculation calves were euthanized; ILN = ileal lymph nodes; JLN = jejunal lymph nodes. TNTC = too numerous to count.

Calf	Treatment	Euthanized**	Ileum	ILN	Jejunum	JLN
2	Library	2	2201*	583	2642	911
4	Library	2	10228*	425	2162	6113*
5	Library	2	392	17	383	44
9	Library	2	27024*	929*	27313*	1649
11	Library	2	1762*	4298*	5527*	4221*
12	Library	2	1178*	397	620*	2465*
15	wt	2	255	428	3080	0
16	wt	2	2527	196	0	85
1	Library	4	22	67	27	711
3	Library	4	124	4255*	602	2813*
6	Library	4	175	443	536	2406*
7	Library	4	0	134	40	217
8	Library	4	409	152	615	482
10	Library	4	158	354	1934	4912*
13	wt	4	12240	8340	14784	6340
14	wt	4	TNTC	5413	TNTC	TNTC

Table 2. Primer pairs for qPCR quantification of select genes.

Gene	Primer	Forward 5'->3'	Reverse Primer 5'->3'
Reference	F57	GTCCCAGGTGTGTTCGAGTT	AATTCCGGTCGAAGGAGTTC
Transposon	MarinerTn	TTCTTCTGAGCGGGACTCTG	GAAATCGGCAAAATCCCTTA
Essential	1082c	CCTGTTCTGATCGCCTCAGT	CATGAACGCAAGAAGGAAGG
Essential	1605c	TCAGCACCAAAACGACTGTG	AAGCTGTTCCACGCCAAG
Essential	3741	CGTTGAGGAGGGCATGTTAT	ATCGAACGCCGTGAAAAG
Essential	4099	GACGGGAAATTCCTTCCCTA	ATCCCAGGTCGACAGGAAC
Nonessential	1466c	TGACCACAGAAACCCAAGTG	ACTACCCCTATGCGCCCTAT
Nonessential	3406c	CTGCAATTGGTCACGAAGC	CCGACGACGTTCAGACTTTC
Nonessential	3065c	GACGTTTCGTGGACAGCAT	CGAACACGGTGAGGAGGTAG

Table 3. Indexed primers used for targeted next generation sequencing.

Pairing was performed with a universal primer from Primer 1 and a uniquely indexed Primer 2 for each sample. Note: Four primer sequences were used for Primer 1 because pooling these staggered primers increased library diversity and yielded better quality scores.

Illumina Sequencing Indexed Primers	Index
Primer 1: Staggered universal adapter:	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGGGACTTATCAGCCAACC	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNCGGGGACTTATCAGCCAACC	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCGGGGACTTATCAGCCAACC	
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNCGGGGACTTATCAGCCAACC	
Primer 2: TruSeq indexed adapter:	
CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	1
CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	2
CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGTCCAGTCTCGCAGATGATAAGG	3
CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGTCCAGTCTCGCAGATGATAAGG	4
CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	5
CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	6
CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGTCCAGTCTCGCAGATGATAAGG	7
CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGTCCAGTCTCGCAGATGATAAGG	8
CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	9
CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	10

Illumina Sequencing Indexed Primers	Index
Primer 2: TruSeq indexed adapter:	
CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGTCCAGTCTCGCAGATGATAAGG	11
CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGTCCAGTCTCGCAGATGATAAGG	12
CAAGCAGAAGACGGCATACGAGAT**TTGACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	13
CAAGCAGAAGACGGCATACGAGAT**GGAACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	14
CAAGCAGAAGACGGCATACGAGAT**TGACAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGTCCAGTCTCGCAGATGATAAGG	15
CAAGCAGAAGACGGCATACGAGAT**GGACGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGTCCAGTCTCGCAGATGATAAGG	16
CAAGCAGAAGACGGCATACGAGAT**GCGGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	17
CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	18
CAAGCAGAAGACGGCATACGAGAT**GGCCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGTCCAGTCTCGCAGATGATAAGG	19
CAAGCAGAAGACGGCATACGAGAT**CGAAAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGTCCAGTCTCGCAGATGATAAGG	20
CAAGCAGAAGACGGCATACGAGAT**CGTACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAGTCTCGCAGATGATAAGG	21
CAAGCAGAAGACGGCATACGAGAT**CCACTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGTCCAGTCTCGCAGATGATAAGG	22

Table 4. *Mycobacterium avium* subsp. *paratuberculosis* monthly shedding results in the feces for individual calves from qPCR analysis.

Shaded boxes were positive for MAP. N/A = calves that were euthanized at 2 mo PI.

Calf	Treatment	1 Mo	2 Mo	3 Mo	4 Mo
1	A1-157L				
2	A1-157L			N/A	N/A
3	A1-157L				
4	A1-157L			N/A	N/A
5	A1-157L			N/A	N/A
6	A1-157L				
7	A1-157L				
8	A1-157L				
9	A1-157L			N/A	N/A
10	A1-157L				
11	A1-157L			N/A	N/A
12	A1-157L			N/A	N/A
13	wt				
14	wt				
15	wt			N/A	N/A
16	wt			N/A	N/A

Table 5. Antibody response measured to MAP antigen by ELISA for calves euthanized at 4 mo PI.

S/P = sample to positive ratio

Calf	Treatment	S/P
1	A1-157L	0.01
3	A1-157L	0.01
6	A1-157L	0.06
7	A1-157L	0.06
8	A1-157L	0.05
10	A1-157L	0.03
13	wt	0.01
14	wt	0.45

Table 6. TA site counts for selected relative quantification genes in the K-10 genome, Wang et al.(2014), and A1-157L.

The raw data from Wang et al. (2014) and the data from the targeted NGS for A1-157L were used to calculated the amount of reads for each TA site that were ≥ 10. TA sites were calculated from the sequenced MAP strain K-10 (GenBank Accession #AE016958.1).

Type	Gene	Length (bp)	TA sites (Gene)	TA Sites Present (Wang et al.)	TA Sites Present (A1-157L)
Essential	1082c	1086	12	1	9
Essential	1605c	834	11	3	8
Essential	3741	846	19	1	11
Essential	4099	1020	11	5	9
Nonessential	1466c	1131	12	1	7
Nonessential	3406c	789	4	2	2
Nonessential	3065c	1302	11	1	1

Table 7. Number of times each gene exhibited an increase or decrease in fold change from the inoculum for all primer pairs in all tissues from relative quantification.

Fold changes were tallied for each gene for all primer pairs for decreases (fold change < 0.90), increases (fold change > 1.10), or no change (between 0.90 and 1.10) compared to the inoculum.

Type	Gene	Decrease	Increase	No Change
Essential	1082c	13	3	0
Essential	1605c	11	5	0
Essential	3741	13	3	0
Essential	4099c	12	3	0
Nonessential	1466c	12	3	0
Nonessential	3406	11	3	1
Nonessential	3065	10	5	1

Table 8. Average fold changes for all disrupted genes from the relative quantification by tissue type.

Intestines = CFUs collected from the ileum and the jejunum. Lymph Nodes = CFUs collected from the ileal lymph nodes and jejunal lymph nodes. F + F = Forward primer and Forward Mar Tn primer; F + R = Forward primer and Reverse Mar Tn primer; R + F = Reverse Primer and Forward Mar Tn; R + R = Reverse primer and Reverse Mar Tn primer. *See Figure 1 for details on primer design.

Tissue Type	**F + F**	**R + F**	**F + R**	**R + R**
Intestines	0.83	2.72	1.65	0.61
Lymph Nodes	1.72	0.97	1.21	1.27

Table 9. Average fold changes for putative essential and nonessential genes by tissue type. The fold changes from All Primer pairs were averaged. Complimentary = primer pairs F + F and R + R. Alternative = primer pairs F + R and R + F. *See Figure 1 for details on primer design.

Gene Type	Tissue Type	All Primer Pairs	Complimentary	Alternative
Essential	Intestines	0.70	0.55	0.86
Essential	LN	1.54	1.83	1.25
Nonessential	Intestines	2.41	0.88	3.96
Nonessential	LN	0.96	1.06	0.87

Table 10. Sum of reads per tissue from the targeted next generation sequencing for the selected genes used for relative quantification. Total reads calculated for all disrupted TA sites for each gene from the targeted NGS for the selected tissues. This includes the total output which is the sum of the reads from all of the tissues.

Note: The following tissues are omitted from this table because the 4-mo euthanasia treatment group was not sequenced: calf 3 ILN and JLN; calf 10 JLN. Inoc Sum = sum of reads in the inoculum; IL = ileum; ILN = ileal lymph nodes; JEJ = jejunum; JLN = jejunal lymph nodes.

Gene	Inoc Sum	2 IL	4 IL	9 IL	11 IL	12 IL	9 ILN	11 ILN	2 JEJ	4 JEJ	9 JEJ	11 JEJ	12 JEJ	4 JLN	9 JLN	11 JLN	12 JLN	Total Output
F57	1009	2225	2324	1127	3	8	14836	131	103	1573	2286	3212	15	10	1473	202	11	29539
MAP1082c	1864	16211	327	9254	92	29	29431	144	526	41	5003	1421	110	44	20357	9980	1116	94086
MAP1605c	2623	145	6	3631	4	5	15640	12	4077	132	6227	25	457	2	9	7	589	30968
MAP3741	2107	8063	15	10	6	18	1	124	0	1	1617	26	4	19	11948	18	38	21908
MAP4099	2806	345	360513	6834	163	217	743	1236	6673	186	8158	117	139	7886	324	908	384	394826
MAP1466c	1583	12401	3290	11247	13	16	43	1744	4253	83	5289	34	42	5552	26	84	26	44143
MAP3406c	2214	452	307	1116	2385	616821	13576	435	813	38	6876	35	1899	378	487	493	615	646726
MAP3065	115	11	9	10	3	15	4	4923	3	2	3	5	5	10	16	11	16916	21946

Table 11. Sum of reads per mouse from the targeted next generation sequencing from Wang et al. (2014) for the selected genes used for the relative quantification.

Total reads calculated for all disrupted TA sites for each gene from the targeted NGS. This includes the total output of the reads from all mice. Note: the sum of reads was calculated from the original raw data from the experiment and not the published additional file.

Gene	Inoc Sum	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Total Output
F57	10168	16830	11453	5571	7554	41408
MAP1082c	4025	3632	3155	2984	1277	11048
MAP1605c	11748	12783	16530	13975	9811	53099
MAP3741	2021920	16640	9852	7205	6902	40599
MAP4099	71652	34338	25654	27376	32813	120181
MAP1466c	26011	46869	23176	26687	26002	122734
MAP3406c	50682	48644	31679	29654	40194	150171
MAP3065	3817	4953	2648	2875	2480	12956

Table 12. Top 42 *in vivo* essential MAP genes.

The top 42 essential genes in MAP as determined from the A1-157L that were absent from all tissues at 2 mo PI.

	Gene Number	Gene Name	Total TA Sites	TA sites in A1-157L	Est. CFUs in A1-157L	Observed CFUs	Expected CFUs recovered from #2	Expected CFUs recovered from #4	Expected CFUs recovered from #9	Expected CFUs recovered from #11	Expected CFUs recovered from #12	H37Rv Homolog	Function
1	MAP1643	aceAb	31	10	31	0	2	6	18	5	1	Rv1916	Isocitrate lyase
2	MAP1531c	MAP1531c	20	9	56	0	4	11	32	9	3	Rv1819c	Probable Drug-transport transmemberane ATP-binding protein ABC transporter
3	MAP1968c	MAP1968c	20	9	25	0	2	5	14	4	1	Rv2224c	Tripeptidyl-peptidase B, Serine peptidase
4	MAP0925	moeA	8	8	55	0	3	10	31	9	3	Rv0994	Probable molybdopterin biosynthesis protein MOEA1
5	MAP0893	uvrD	25	7	19	0	1	4	11	3	1	Rv0949	ATP-dependent DNA helicase PcrA
6	MAP1506	MAP1506	22	7	42	0	3	8	24	7	2	Rv1789	PPE Family protein
7	MAP4116c	mmaA4	15	7	35	0	2	7	20	6	2	Rv0642c	Methoxy mycolic acid synthase 4 MMAA4
8	MAP0945c	pabB	12	7	20	0	1	4	11	3	1	Rv1005c	Para-aminobenzoate synthase component I
9	MAP1505	MAP1505	19	6	17	0	1	3	9	3	1	Rv1706c	PPE Family protein
10	MAP3851c	MAP3851c	15	6	42	0	3	8	24	7	2	Rv0385	Hypothetical protein
11	MAP3244	MAP3244	12	6	15	0	1	3	8	2	1		DNA topoisomerase
12	MAP3862c	MAP3862c	9	6	31	0	2	6	17	5	1	Rv0364	Possible conserved transmembrane protein
13	MAP2434	glgB	38	5	18	0	1	3	10	3	1	Rv1326c	Glycogen branching enzyme
14	MAP1634	MAP1634	24	5	15	0	1	3	9	2	1		Transcriptional regulator (LysR)

	Gene Number	Gene Name	Total TA Sites	TA sites in A1-157L	Est. CFUs in A1-157L	Observed CFUs	Expected CFUs recovered from #2	Expected CFUs recovered from #4	Expected CFUs recovered from #9	Expected CFUs recovered from #11	Expected CFUs recovered from #12	H37Rv Homolog	Function
15	MAP0877c	MAP0877c	17	5	15	0	1	3	9	2	1		Mannitol 2-dehydrogenase
16	MAP1548c	MAP1548c	15	5	35	0	2	7	20	5	2	Rv1836c	Hypothetical protein
17	MAP1267	MAP1267	13	5	16	0	1	3	9	2	1		Transcriptional regulator (Gntr)
18	MAP3884	MAP3884	13	5	14	0	1	3	8	2	1	Rv0407	Probable F420-Dependent Glucose-6-phosphate dehydrogenase FGD1
19	MAP2060c	MAP2060c	12	5	14	0	1	3	8	2	1		AcetylCoA acetyltransferase
20	MAP3608	lprK	12	5	13	0	1	2	7	2	1	Rv0173	Possible MCE-Family lipoprotein LPRK
21	MAP1148	MAP1148	8	5	20	0	1	4	11	3	1	Rv1422	Hypothetical protein
22	MAP1908	MAP1908	8	5	15	0	1	3	8	2	1	Rv2170	Hypothetical protein
23	MAP0968	MAP0968	5	5	37	0	2	7	21	6	2		Transcriptional regulator (TetR)
24	MAP0294c	pca	38	4	14	0	1	3	8	2	1	Rv2967c	Probable pyruvate carboxylase PCA
25	MAP1233	MAP1233	31	4	12	0	1	2	7	2	1	Rv1513	Hypothetical protein
26	MAP1335	uvrB	29	4	11	0	1	2	6	2	1	Rv1633	Excinuclease ABC subunit B
27	MAP1385c	MAP1385c	24	4	8	0	0	1	4	1	0		Glucose-methanol-choline oxidoreductase
28	MAP3744	MAP3744	22	4	17	0	1	3	10	3	1		Thiazolinyl imide reductase
29	MAP1463	MAP1463	21	4	9	0	1	2	5	1	0		Cyclohexanone monooxygenase
30	MAP0081	MAP0081	19	4	28	0	2	5	16	4	1	Rv0063	Possible oxidoreductase
31	MAP0765	MAP0765	19	4	15	0	1	3	9	2	1		MCE Family protein
32	MAP2767c	MAP2767c	17	4	23	0	1	4	13	4	1		Hypothetical protein
33	MAP0410	dppB	14	4	11	0	1	2	6	2	0	Rv3665c	Probable Dipeptide-transport integral membrane protein ABC transporter DPPB

	Gene Number	Gene Name	Total TA Sites	TA sites in A1-157L	Est. CFUs in A1-157L	Observed CFUs	Expected CFUs recovered from #2	Expected CFUs recovered from #4	Expected CFUs recovered from #9	Expected CFUs recovered from #11	Expected CFUs recovered from #12	H37Rv Homolog	Function
34	MAP3873	metZ	14	4	11	0	1	2	6	2	0	Rv0391	O-succinylhomoserine sulfhydrylase
35	MAP2562	MAP2562	12	4	17	0	1	3	10	3	1	Rv1216c	Probable conserved integral membrane protein
36	MAP0757	MAP0757	9	4	20	0	1	4	11	3	1	Rv3501c	Conserved hypothetical integral membrane protein YRBE4A
37	MAP0153	MAP0153	8	4	40	0	3	8	23	6	2	Rv0902c	Two Component sensor histidine kinase PRRB
38	MAP0190	glpQ1	8	4	19	0	1	4	11	3	1	Rv3842c	Probable glycerophosphoryl diester phosphodiesterase GLPQ1
39	MAP0969	cprA	8	4	22	0	1	4	12	3	1		Transcriptional regulator
40	MAP3311c	MAP3311c	8	4	6	0	0	1	3	1	0	Rv3210c	Hypothetical protein
41	MAP0357	MAP0357	7	4	13	0	1	3	8	2	1	Rv3694c	Possible conserved transmembrane protein
42	MAP2539c	lpqZ	6	4	29	0	2	5	16	5	1	Rv1244	Probable lipoprotein LPQZ

Note: Total TA sites = total number of TA sites for each gene determined from the K-10 genome. TA sites in Inoc. = number of TA sites disrupted in the inoculum. Est. CFUs = the estimated number of CFUs calculated for all TA sites for each gene in the 100,000 CFU library from A1-157L. Observed CFUs = Estimated CFUs using reads from the targeted NGS for the inoculum and the total colony count for each sample. Expected CFUs Recovered was calculated using the ratio of the number of reads in the inoculum to the total number of TA sites in the MAP genome which was then calculated for the total number of CFUs recovered from each animal.

Table 13. Enrichment analysis from Gene Ontology (GO).

GO Biological Process	Essential Genes	Fold Enrichment	P-value Corrected
DNA Repair	18	3.24	0.01
Pathogenesis	26	2.34	0.04
Symbiosis and Parasitism	43	2.02	0.01
Growth	110	1.83	0.0000001
Response to Stimulus	77	1.77	0.0004
Oxidation-reduction Process	75	1.62	0.01

Table 14. Top *in vivo* genes from the A1-157L in cattle compared to the K-10 genes reported in mice (Wang et al., 2014).

	A1-157L top genes	**Protein Function in *M. tuberculosis***	**Wang et al., 2014**	**Protein Function in *M. tuberculosis***
1	MAP1643	Isocitrate lyase	MAP0298	Probable transcriptional regulator protein
2	MAP1531c	Probable drugs-transport transmembrane ATP-binding protein ABC transporter	MAP0704	Probable cytochrome P450 141 Cyp141
3	MAP1968c	Tripeptidyl-peptidase B. Serine peptidase. MEROPS family S33	MAP0865	ESX conserved component EccC3 ESX-3 type VII
4	MAP0925	Probable molybdopterin biosynthesis protein MOEA1	MAP0908c	Hypothetical protein
5	MAP0893	ATP-dependent DNA helicase PcrA (EC 3.6.1.-)	MAP0977	Putative polyketide synthase Pks16
6	MAP1506	PPE Family protein	MAP1031c	Probable spermidine synthase SpeE
7	MAP4116c	Methoxy mycolic acid synthase 4 MMAA4 (methyl mycolic acid synthase 4)	MAP1082c	Possible monooxygenase
8	MAP0945c	Para-aminobenzoate synthase component I	MAP1195c	Probable acyl-CoA dehydrogenase FadE15
9	MAP1505	PPE Family protein	MAP1236c	Probable daunorubicin-dim-transport integral membrane protein ABC transporter DrrC
10	MAP3851c	Hypothetical protein	MAP1576	Hypothetical protein
11	MAP3244	DNA topoisomerase	MAP1584c	Hypothetical protein
12	MAP3862c	Possible conserved transmembrane protein	MAP1601	n.a.
13	MAP2434	Glycogen branching enzyme (EC 2.4.1.18)	MAP1605c	Probable short-chain type dehydrogenase/reductase
14	MAP1634	Transcriptional regulator (LysR)	MAP1822c	Hypothetical alanine and proline rich protein
15	MAP0877c	Mannitol 2-dehydrogenase	MAP1835c	Proteasome beta subunit PrcB
16	MAP1548c	Hypothetical protein	MAP1914	Probable transmembrane serine/threonine-protein kinase
17	MAP1267	Transcriptional regulator (Gntr)	MAP2008	L PknL (protein kinase L) S-nitrosomycothiol reductase MscR
18	MAP3884	Probable F420-dependent glucose-6-phosphate dehydrogenase FGD1	MAP2385c	Probable acyl-CoA dehydrogenase FadE29

19	MAP2060c	AcetylCoA acetyltransferase	MAP2439c	Hypothetical protein
20	MAP3608	Possible MCE-family lipoprotein LPRK	MAP2582c	Hypothetical protein
21	MAP1148	Hypothetical protein	MAP2964c	Probable integrase/recombinase
22	MAP1908	Hypothetical protein	MAP3070	Probable dehydrogenase
23	MAP0968	Transcriptional regulator (TetR)	MAP3131	Probable conserved transmembrane transport protein MmpL4
24	MAP0294c	Probable pyruvate carboxylase PCA	MAP3327c	Hypothetical protein
25	MAP1233	Hypothetical protein	MAP3352c	Probable transcriptional regulatory protein
26	MAP1335	Excinuclease ABC subunit B	MAP3420c	PPE family protein PPE22
27	MAP1385c	Glucose-methanol-choline oxidoreductase	MAP3699c	Probable succinate dehydrogenase [membrane anchor subunit]
28	MAP3744	Thiazolinyl imide reductase	MAP3951c	Probable peptidase
29	MAP1463	Cyclohexanone monooxygenase	MAP4117c	Methoxy mycolic acid synthase 1
30	MAP0081	Possible Oxidoreductase		
31	MAP0765	MCE family protein		
32	MAP2767c	Hypothetical protein		
33	MAP0410	Probable dipeptide-transport integral membrane protein ABC transporter DPPB		
34	MAP3873	O-succinylhomoserine sulfhydrylase		
35	MAP2562	Probable conserved integral membrane protein		
36	MAP0757	Conserved hypothetical integral membrane protein YRBE4A		
37	MAP0153	Two component sensor histidine kinase PRRB		
38	MAP0190	Probable glycerophosphoryl diester phosphodiesterase GLPQ1 (Glycerophosphodiester phosphodiesterase)		
39	MAP0969	Transcriptional regulator		
40	MAP3311c	Hypothetical protein		
41	MAP0357	Possible conserved transmembrane protein		
42	MAP2539c	Probable lipoprotein LPQZ		

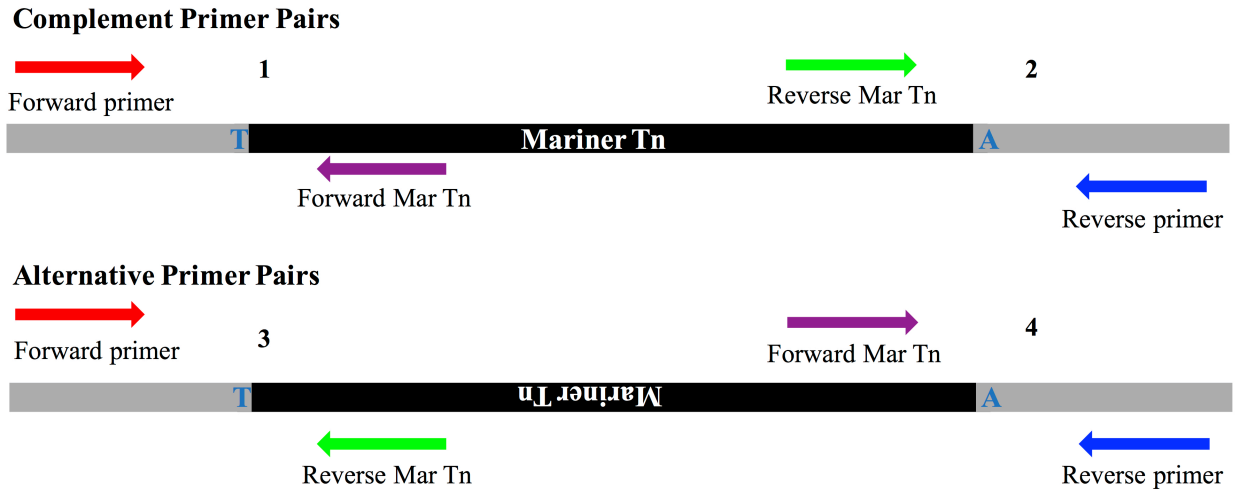
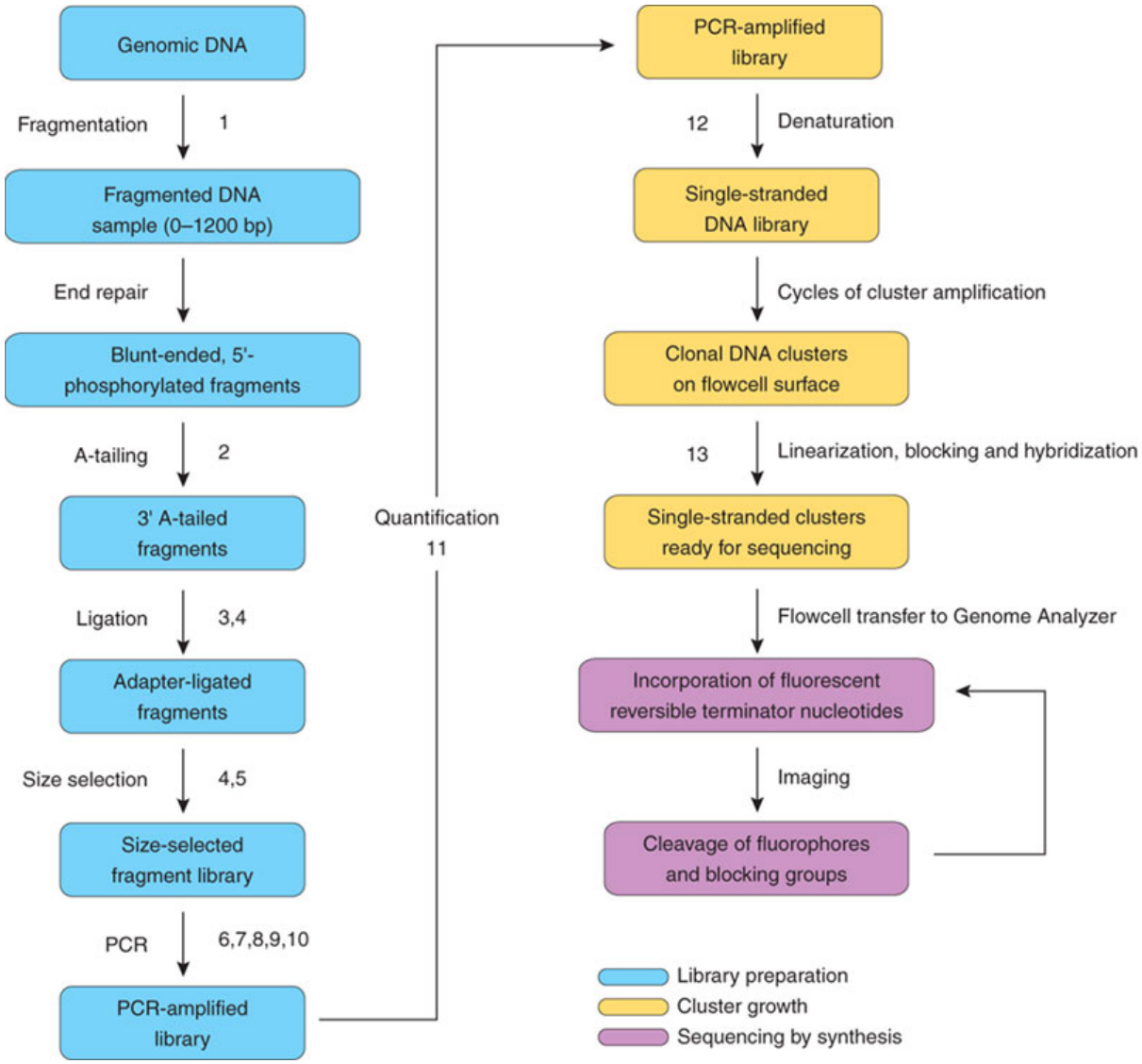


Figure 1. Primer pairs for qPCR quantification of selected genes.

Each number indicates a different set of primers used for each gene. Four sets of primers were used to account for instances in which the mariner transposon was inserted in an inverted position in the TA site of the gene.

Figure 2. Workflow for preparation and sequencing genomic DNA for targeted NGS.



(Quail et al., 2008)

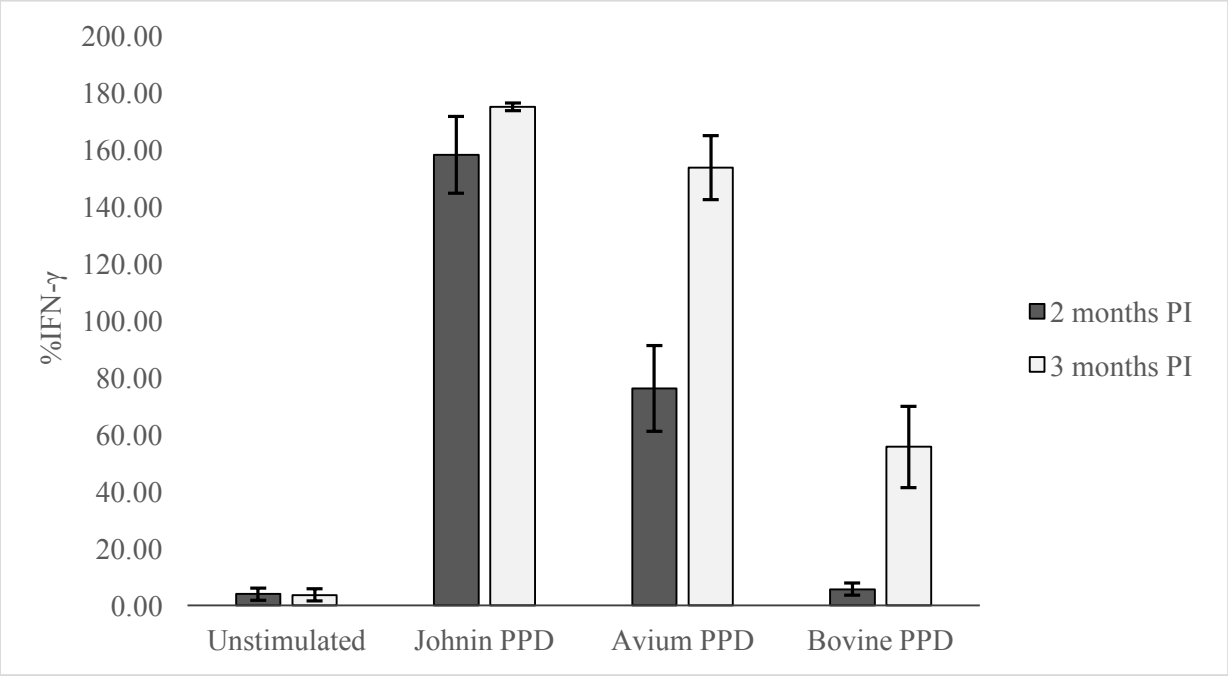


Figure 3. Average% IFN-γ from all calves euthanized at 2 and 3 mo post inoculation (PI).

The average %IFN-γ from all calves was calculated for each of the time points.

Note: 8 calves were euthanized at 2 mo post inoculation (6 A1-157L, 2 A1-157 wt), therefore, only 8 calves were sampled at 3 mo of age.

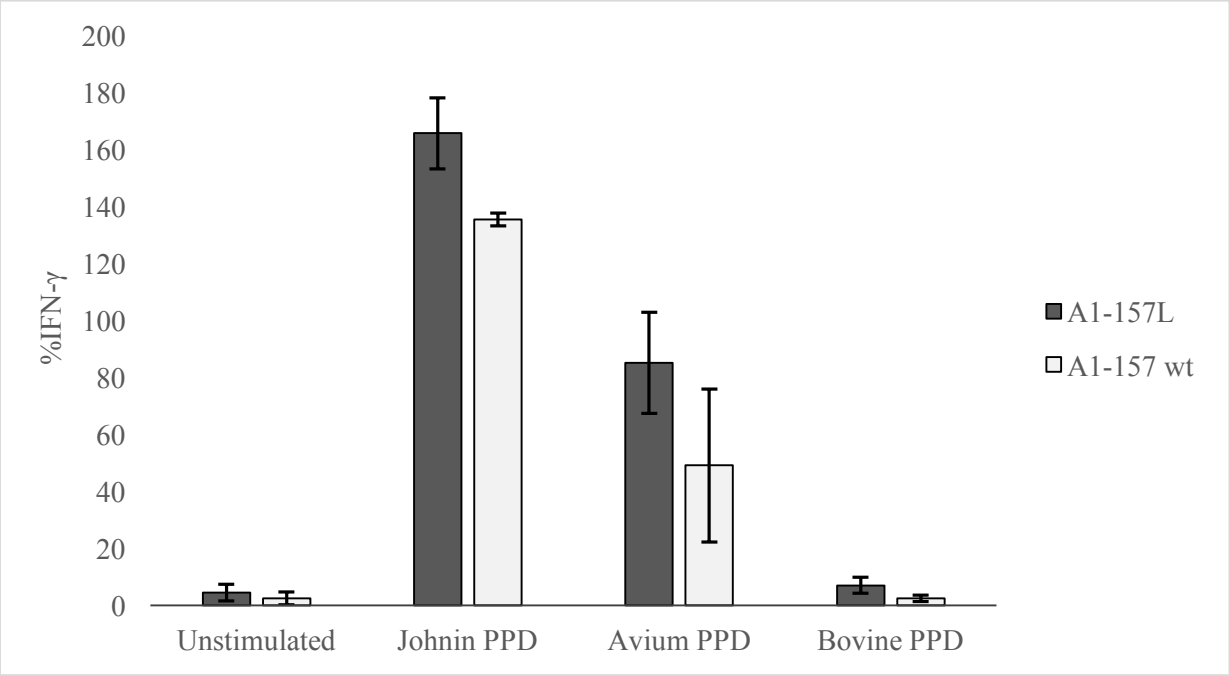


Figure 4. Average %IFN-γ production from A1-157L and A1-157 wt calves at 2 mo PI.

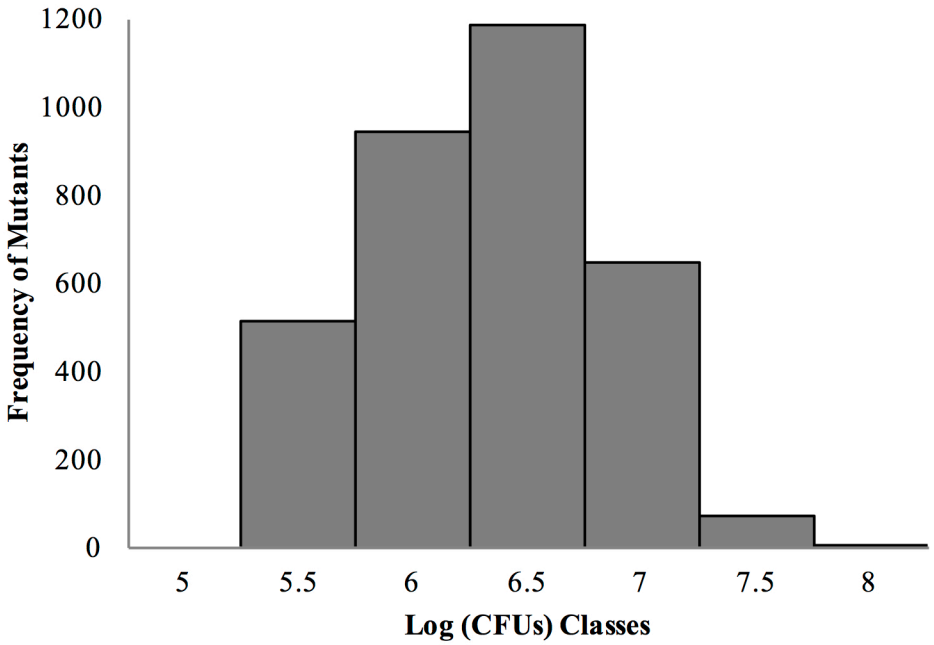


Figure 5. Frequency of TA dinucleotide mutants present in the inoculum at different log values. This histogram represents the frequency of mutants at different log values in the inoculum (10^{11} CFUs) administered to each calf.

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