STRAIN DEPENDENT VARIATIONS IN IRON METABOLISM OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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Dedication

To my lovely wife, Madhu

Abstract

Johne's disease is a major animal health problem of ruminant species worldwide and imposes significant economic losses to the industry. Our ability to culture the causative agent--*Mycobacterium avium* subsp. *paratuberculosis* (MAP)--and therefore its rapid diagnosis and our understanding of its virulence is limited. MAP is difficult to culture because of its unusually strict iron requirements. For optimal growth in laboratory media, MAP requires a siderophore (mycobactin) supplementation that makes MAP fastidious, often requiring eight to sixteen weeks to produce colonies in culture – a major hurdle in timely diagnosis and therefore implementation of optimal control measures. Understanding iron regulatory networks in the pathogen in vitro is therefore of great importance.

Several microbiological and genotyping studies and clinical observations suggest that Johne's in certain hosts such as sheep, goats, deer, and bison is caused by a distinct set of strains that show a relatively high degree of host preference. At least two microbiologically distinct types of MAP have been recognized. A less readily cultivable type is the common, but not invariable, cause of paratuberculosis in sheep (type I), while another readily cultivable type is the most common cause of the disease in cattle (type II). In addition, since the MAP genome sequence was published in 2005, very little research has focused on iron physiology and its contribution to metabolic networks of this fastidious organism.

Based on these observations, I **hypothesize** that iron dependent gene regulation is different between type I and type II MAP strains.

Iron dependent Regulator (IdeR), a transcription factor, is an essential gene in MAP and differentially controls the expression of genes involved in iron physiology in the two strain types of MAP. We identified polymorphisms in the IdeR open reading frame (ORF) and the promoters of putative IdeR regulated genes between the type I and type II strains of MAP. Structure-function association studies revealed repression of an iron storage gene, *bfrA* in the presence of iron by type I MAP strain alone. In contrast, *bfrA* was upregulated in the presence of iron in type II MAP strain. This leads us to propose that type I MAP strains may experience iron toxicity when excess iron is provided in the medium. The rationale is that excess free iron is detrimental to the cells and must be stored in bacterioferritins, a feature that type I strains lack.

Transcriptional and proteomic profiling of these MAP strains under iron-replete or –deplete conditions revealed that iron-sparing response to iron limitation was unique to the type II strain as evidenced by repression of non-essential iron utilization enzymes (aconitase and succinate dehydrogenase) and upregulation of proteins of essential function (iron transport, [Fe-S] cluster biogenesis and cell division). Under iron-replete conditions, type II MAP alone increased expression of BfrA (bacterioferritin) and MhuD (mycobacterial heme utilization, degrader) protein, which is intricately involved in iron recycling. These findings further supported the contention that type I MAP strains are metabolically inept under iron-replete conditions.

The intracellular lifestyle of MAP in the intestines and lymph nodes of natural infection revealed that MAP deployed genes involved in maintaining iron homeostasis under iron stress in the tissues of infected animals. There was a clear dichotomy in in

vitro infected macrophages and natural infection in the expression profiles of both iron acquisition genes and other virulence factors involved in MAP survival inside the host.

In summary, our studies revealed that IdeR of type II strain regulates mycobactin synthesis and iron storage genes, similar to the function of IdeR in *M. tuberculosis* (MTB), while the type I strain is deficient in iron storage function. Given our inability to delete ideR, it appears that this is an essential gene (as in MTB) for MAP survival. MAP IdeR regulon studies led us to define a novel operon carrying genes encoding a potential secretory apparatus (ESX-3/type VII secretory system). Functional analysis of the iron-induced proteome also identified novel ESAT-6 (early secreted antigenic target) family of proteins belonging to ESX-5, which have been identified as major virulence factors in MTB. We also established that, type I MAP strains are more sensitive to fluctuations of environmental iron due to defective regulation of *bfrA* and may grow better under lower iron levels in the culture media. Taken together, our studies suggest that MAP employs a sophisticated repertoire of proteins that are inter-connected and function in response to environmental stress.

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CHAPTER 1: INTRODUCTION

The genus *Mycobacterium* belongs to the phylum *Actinobacteria,* which is one of the largest taxonomical units within the domain bacteria (42). *Actinobacteria* include plant commensals (*Leifsonia* spp.), soil dwellers (*Streptomyces* spp.) and pathogens (*Corynebacterium* and *Mycobacterium*). They exhibit various morphological features, coccoid (*Micrococcus*) or rod-coccoid (*Arthrobacter*) to highly differentiated mycelia (*Streptomyces*) (34). Furthermore, they have unusual developmental features such as the spore forming nature of *Streptomyces* or the ability to persist in a non-replicating state as seen in some pathogenic mycobacteria. Principal genetic events such as gene duplication, gene decay, horizontal gene transfer (HGT), and genomic rearrangements have shaped actinobacterial genera including mycobacteria (2, 17, 20, 38). Existence of several functional classes of genes belonging to fatty acid metabolism and multiple copies of a type VII secretory system in mycobacteria are classic examples of gene duplication (4, 8, 37). HGT and genomic rearrangements are also reported in mycobacteria (21, 22, 44, 49).

Mycobacterial genomes are GC rich (up to 70%) and currently over 100 species and over 10 subspecies of mycobacteria (46)(J.P. Euzeby, *List of Prokaryotic Names with Standing in Nomenclature* [http://www.bacterio.cict.fr]) have been identified. The most common mycobacterial species that significantly impact on human and animal health include members of *M. tuberculosis* Complex (MTBC), *M. leprae*, and *M. avium. M. avium* consists of several subspecies collectively called the *M. avium* Complex (MAC) (33, 43, 46, 50). Data from epidemiological, taxonomical and comparative genomic

studies show that MAC represents a microcosm of genotypically and phenotypically diverse mycobacteria that have variations in host preference.

M. avium subsp. *avium* (MAA), primarily causes avian tuberculosis, and is the cause of systemic infection in immune compromised humans. *M. avium* subsp. *hominissuis* (MAH), which is genetically distinct from MAA (3, 19) is most commonly associated with pulmonary infections in pigs and humans. *M. avium* subsp. *silvaticum* (MAS) causes TB-like lesions in wood pigeons (24). *M. avium* subsp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease (JD) in ruminant livestock worldwide; however, strict host adherence does not occur as MAP has been isolated from other mammalian species including humans (5, 14, 15). Furthermore, there is also substantial evidence of the existence of two strains of MAP – cattle (type II) and sheep (type I) that have genotypic and phenotypic diversity (27).

Comparative genomic hybridizations, short sequence repeat analysis and single nucleotide polymorphisms of MAP isolates obtained from diverse host species have established and indexed genomic differences between types I and II strains of MAP (25, 28-31, 35, 36). Phylogenetic analysis of sequences has identified type I and type II strains as separate pathogenic clones that share a common ancestor (1, 44, 45). Furthermore, in vitro cellular studies show distinctive host response phenotypes between the two MAP strain types (18, 26).

Differences between type II and I strains are also demonstrated in the clinical manifestation of JD in cattle and sheep (6, 7). While JD is characterized as a subclinical infection with mild to moderate pathology in sheep, infection in cattle in manifested as

chronic progressive disease with moderate to severe pathology. Unlike cattle, sheep infected with MAP do not exhibit profuse diarrhea and overt thickening of the intestines. This disease state is reminiscent of lepromatous leprosy in humans associated with paucibacillary pathology, which is also the case in sheep JD due to the difficulty to confirm the presence of MAP in intestines by acid fast staining. The differences observed in the clinical and pathological manifestation of the disease in cattle and sheep may be influenced by MAP strain and/or the differences in host immune response.

Animals primarily acquire infection via fecal-oral route. MAP must first overcome host defense mechanisms operated at the intestinal interface before it can successfully establish an infection in its principle cell type, the macrophage. MAP deploys a myriad of defenses to combat intracellular oxidative stress, low pH, and nutrient starvation to replicate and persist inside the host for prolonged periods of time (12, 39-41).

Animals can remain infected for several years (2-3 years) and excrete MAP in the feces without developing clinical signs of the disease. Infected animals intermittently shed the organisms into the environment, serving as a source of infection for other animals. The existence of supershedders that shed trillions of organisms per gram of feces has been recently shown. However, rapid detection of sub-clinically infected animals and Johne's disease control strategies still pose a major challenge (9-11).

Rapid detection of infected animals is crucial for successful execution of a Johne's disease control program. The routine practice for unambiguous detection of MAP infection is culturing the organism from the feces of suspected animals. MAP is difficult

to culture because of its unusually strict iron requirements (16). For optimal growth in laboratory media, MAP requires siderophore (mycobactin) supplementation that makes MAP fastidious, often requiring eight to sixteen weeks to produce colonies in culture – a major hurdle in timely diagnosis and therefore implementation of optimal control measures. Furthermore, sheep MAP strains take even longer to grow compared with cattle MAP strains. Therefore there is an urgent need to make continuous refinements to our current detection methods.

Iron is considered both as an essential nutrient and as a major virulence factor (13). Thus, augmenting our knowledge of MAP iron physiology is critical to identify virulence attributes and tailor in vitro culture methods. The **overall objective** of the studies presented in this thesis is to elucidate iron dependent gene regulation and delineate the differences between the two strain types of MAP. This thesis is organized into the following chapters. **Chapter 2** provides a focused literature review on iron metabolism in eukaryotes and prokaryotes with emphasis on mycobacteria. **Chapters 3** and **4** provide the evidence for accepting my hypothesis that iron dependent gene regulation is different between type I and type II MAP strains. Most bacteria, including MTB, employ a transcriptional factor to regulate expression of genes involved in maintaining iron homeostasis (32, 47). In **chapter 3** I characterized the role of IdeR, a transcriptional regulator, in the two diverse strains of MAP. Iron is critical for the activity of several key enzymes. Bacteria use multiple mechanisms to modulate iron acquisition and storage (23), including pathways independent of IdeR (48). Hence, in **chapter 4** I profiled the global transcriptome and proteomes of the two MAP strains to identify gene-

protein correlates in response to iron. A systems analysis of transcriptomes in lesions of naturally infected cattle and in vitro infection of macrophages confirmed our iron-regulated responses. These findings are reported in **chapter 5**. **Chapter 6** summarizes the findings presented in this thesis in light of its contribution to our overall understanding of the iron physiology of MAP and proposes future studies.

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CHAPTER 2: LITERATURE REVIEW

1.1 Significance of iron:

Although iron is one of the most abundant materials on earth and required only in micromolar concentrations by cells, it is inaccessible in biological environments (43). The most stable forms of iron are Fe^{3+} and Fe^{2+}. In aerobic inorganic environments, the ferric form of iron (Fe^{3+}) aggregates into oxy-hydrite crystals (4, 10, 159). When reduced, Fe^{2+} activates the Fenton reaction ($Fe^{2+} + H2O2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$) leading to formation of hydroxyl radicals that are detrimental to cells. Therefore iron needs to be sequestered into carrier proteins in order to protect cells from oxidative damage (7, 60).

Iron is a cofactor in several enzymatic reactions due to its high redox potential (9, 25). Central to this are enzymatic reactions involved in electron transport, FeS cluster biogenesis, nucleic acid synthesis and oxidative stress response (49). For example, riboreductases that convert ribose (RNA) to deoxyribose sugars (DNA) require iron as a catalyst. Therefore iron is critical for most living systems. However, pathogens such as *Borrelia burgdoferi*, the causative agent of Lyme disease and the soil bacterium *Lactobacillus plantarum* have bypassed the need for iron (96, 149). Regardless, iron due to its important roles in diverse cellular activities is considered an essential nutrient by most living cells.

1.2 Iron at host-pathogen interface:

The combat between host and pathogen for iron is an exciting phenomenon that involves tactics from both the host and the pathogen. The physiological levels of iron in the host (10^{-18}M) are far below those required to support the growth of most pathogens (10^{-6}M to 10^{-7}M) (108, 145). To limit infection, host cells sequester iron by upregulating iron carrying proteins such as lactoferrin, transferrin and ferritin. However, pathogens produce iron-chelating compounds called siderophores that have higher affinity for iron than lactoferrin (28, 90). In response, host cells produce lipocalin-2 (Lcn2), a secreted glycoprotein, that binds to siderophores and abrogates iron uptake by the pathogen (59, 62, 105). In order to counterbalance the deleterious effects of Lcn2, some bacterial pathogens produce a glycosylated siderophore called salmochelin that sequesters Lcn2 (1, 32, 33, 128). Other pathogens, like *M. avium,* traffic inside the endosome in order to avoid contact with Lcn2 (48). A fundamental tenet that arises from these observations is that the pathogen must have efficient iron acquisition systems for successful colonization in the host. However, excess free iron can generate hydroxyl radicals that are extremely toxic to cells (125). This warrants that iron acquisition and storage systems in bacteria are constantly monitored. Since they contribute significantly to the survival in the host, iron regulatory elements of pathogenic bacteria have been deemed virulence factors.

1.3 Iron uptake and metabolism in multicellular eukaryotes:

Mammals obtain iron exclusively from their diet. Iron absorption takes place in the proximal small intestine near the gastro-duodenal junction (102). The specialized

membrane transporter protein, duodenal cytochrome *b* (DCYTB), functions as a ferric reductase to reduce dietary nonheme iron and transports iron across the gut epithelium (64, 88). The expression of DCYTB increases in response to iron deficiency. Fe^{+2} is transported across the cellular membrane by divalent metal transporter 1 (DMT1, also known as SLC11A2, NRAMP2, and DCT1) (21). Heme iron is transported across the gut lumen into enterocytes via heme carrier protein 1 (81). Later, iron is released from heme via the action of hemeoxygenase and stored in ferritin within the enterocytes. During iron demand, basolateral transfer of iron into circulation occurs via a specialized membrane transport protein called, ferroportin (FPN, also known as SLC40A1, IREG1, and MTP1). FPN plays a critical role in export of iron out of enterocytes and for export of iron from tissue macrophages (44). Intestinal epithelial cells and macrophages are the major cellular stores for iron. When iron demand is reduced, hepcidin secreted from the liver binds FPN and causes its internalization and degradation (85, 153). Subsequently iron accumulates within the enterocytes and is stored in the form of ferritin and is lost when the enterocytes migrate up the villus and are sloughed into the intestinal lumen. This enables the enterocytes to buffer against excessive iron absorption.

Iron within the plasma is bound to a protein called transferrin (2, 164). Each transferrin can bind two atoms of ferric iron creating ferric transferrin, which is the most efficiently utilized iron form by cells. Ferric transferrin binds to the transferrin receptor (TfR1) on cells to deliver the iron cargo via endocytosis (3). TfR1 is expressed on most cells and TfR1 deficiency leads to embryonic lethality in mice suggesting its importance in iron metabolism in eukaryotes (75). The TfR1-diferric transferrin complex is

endocytosed by clathrin-coated pits and the resulting vesicles are then uncoated to become endosomes (103). Vacuolar ATPase acidifies the endosomes at a pH of 5.5 and iron is released from transferrin.

Posttranscriptional regulation of iron utilization proteins is mediated via RNA-binding iron regulatory proteins (IRPs) (47, 84). When cellular iron levels are low, iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2) bind to stem loop structures, known as iron responsive elements (IREs) in the mRNAs of iron metabolism proteins (53, 57). If IRE is located in the 5' untranslated region (UTR), the binding of IRPs block translation, however, stabilization of mRNA occurs if IRE is in the 3' UTR. TfR1 mRNA has IRE in the 3' UTR whereas ferritin mRNA has IRE in the 5' UTR. When iron requirements are high, binding of IRP stabilizes *tfR1* and blocks translation of ferritin mRNA. This increases iron uptake and reduces iron storage. *dmt1* has IRE in its 3' UTR whereas *fpn* has IRE in the 5' UTR.

1.4 Iron uptake and metabolism in unicellular eukaryotes:

Pathogenic unicellular eukaryotes such as *Candida albicans, Trichomonas foetus, Trypanosoma brucei and Leishmania spp.* have developed sophisticated systems to acquire iron from the host (132).

The non-pathogenic yeast, *Saccharomyces cerevisiae* has been studied as a model to understand eukaryotic iron metabolism. *S. cerevisiae* can survive under extremely scarce or abundant iron environments (94). To withstand these vast fluctuations in iron availability, *S. cerevisiae* employs a tight transcriptional and posttranscriptional

regulation. The iron dependent transcriptional factor Aft1p regulates gene expression involved in iron physiology (13). Aft1p is constitutively expressed and under iron-replete conditions, is localized in the cytosol so it does not activate transcription (161). However, under iron-deplete condition, Aft1p localizes to the nucleus, binds to DNA and activates transcription. Aft1p is translocated to the nucleus via a karyopherin Pse1p (137). Aft1p regulates expression of at least seventeen genes that are involved in iron uptake such as siderophore binding, siderophore reductases, metalloreductases, and enterobactin transport and heme oxygenase (115, 124). Transport of ferric siderophore is facilitated by cell wall mannoprotein system called FIT (ferric iron transport) regulated by Aft1p (97).

Interestingly, *S. cerevisiae* and other yeast species do not express the iron storage protein, ferritin found in other eukaryotes and prokaryotes. Instead under iron rich environments, they accumulate iron inside the vacuoles via the action of a transporter protein called Ccc1p (68, 74). In addition to vacuolar iron storage pool, iron is incorporated into heme and iron sulfur clusters in mitochondria (101). Sequestration of iron within the vacuoles or iron sulfur clusters protects the cells from cytotoxic effects of excess iron. Under iron deplete conditions, *Ccc1* transcription is shut off and Aft1p directs expression of other proteins to allow the efflux of iron from the vacuole into the cytosol (126). Heme oxygenase (Hmx1p) expression under iron deficiency ensures degradation of heme and recycling iron for other cellular activities (63).

C. albicans, the causative agent of thrush in humans uses a reductive iron uptake mechanism (5, 65, 104). *C. albicans*, is incapable of siderophore production and must use a specific membrane protein (Arn 1 or Sit 1) to acquire iron (46). Plasma membrane

ferrireductases (Fre1 and Fre2) aid in the reduction of Fe^{3+} to Fe^{2+} from a wide variety of ferric complexes (6). *C. albicans* can also acquire haem and transferrin bound iron within the host (118, 150). Some non-pathogenic fungi such as *Schizosaccharomyces pombe* produce siderophores; therefore, production of siderophores alone is not intricately linked to pathogenesis (46).

Tritrichomonas foetus, the causative agent of sexually transmitted disease-bovine trichomoniasis has high requirements for iron (50-100uM) (133). *T. foetus* can take up iron from lactoferrin or transferrin. Iron uptake from lactoferrin is mediated via endocytosis, while uptake from transferrin occurs via extracellular release (134). *T. foetus* can also acquire iron via siderophores and by breakdown of heme (69, 70).

Trypanosoma brucei causes sleeping sickness in humans and domestic livestock in Africa. *T. brucei* efficiently acquires transferrin bound iron in the flagellar pocket via surface receptors encoded by *ESAG-6* and *ESAG-7* (93, 130). These genes are located on a poly-cistronic transcription unit called the variant surface glycoprotein (VSG) that forms the surface coat of the parasite. *T. brucei* has several VSG expression sites, only one of which is active at each time, and each of these VSG expression sites carry a functional copy of *ESAG-6* and *ESAG-7.* This provides a fitness advantage to the parasite as it can alter affinities of *ESAG-6* and *ESAG-7* mediated receptors to transferrin from various host species (12).

One of the intriguing features of unicellular eukaryotic iron metabolism is iron storage. Iron must be stored in a complex with iron binding protein in order to avoid generation of reactive oxygen species. In other multicellular eukaryotes and bacteria this

is achieved by the iron storage protein, ferritin (51). Ferritin is a spherical shell-like protein, consisting of 24 subunits assembled into a hollow sphere that has binding sites for several Fe^{3+} ions (119). Iron temporarily stored in ferritins is released under iron-deprivation. The majority of unicellular eukaryotes do not possess ferritin therefore iron storage mechanisms in these organisms are not clear.

1.5 Iron uptake in bacteria:

Iron is a critical factor in determining the ability of a pathogen to establish successful infection (8, 106, 108). To defend pathogens, host presents either an iron deficient environment or uses excess iron to generate reactive oxygen species (91, 148). In vertebrate hosts following dietary assimilation, iron is coupled to a transport protein called transferrin and mobilized into various cells. Transferrin has a surplus iron binding capacity that ensures non-availability of free iron to pathogens resulting in a key defense mechanism against bacterial replication (22). It has been shown that supplementing iron during bacterial infections aggravates disease condition (148). Another defense tactic employed by the host is to elevate iron levels in phagosomes located within macrophages (168). Following internalization of the pathogen, natural resistance associated macrophage proteins (Nramp1) up regulates iron transport into the phagosome and generates reactive oxygen species (ROS). Despite these host defense mechanisms, the pathogen prevails. Several pathogens like *Salmonella, Listeria, and Mycobacteria* have evolved mechanisms to reside within macrophages and sequester iron from the host (159,

160, 165, 167). Bacteria can either come in direct contact with iron sources or release siderophores in order to meet iron requirements.

1.5.1 Direct assimilation of ferrous iron:

Bacteria living in anaerobic or reducing environments can come in direct contact with Fe^{2+} that diffuses through the outer membrane porins of gram-negative bacteria (15, 61, 140). It is transported through the cytoplasmic membrane via the ABC ferrous iron transporter present in several bacterial species. For example, *Helicobacter pylori* that colonize the stomach can directly assimilate iron through FeO system. Following cell surface contact, ABC transporter proteins further facilitate mobilization of iron into the cell. Bacterial species such as *Neisseria meningitides* and *N. gonorrhoeae* have transferrin and/or lactoferrin uptake systems. Ferritins also serve as a source of iron for pathogens such as *Listeria monocytogenes* and *Pasteurella multocida (27).* Receptors HemR of *Yersinia enterocolitica* and HmbR of *Neisseria meningitidis* sequester iron bound to hemoglobin by hemolysis (114, 147). Haptoglobin is a glycoprotein that binds to hemoglobin released by hemolysis for clearance by the liver (83). HpuA of *N. meningitides* serves as a surface receptor for this complex and hence sequesters iron (71, 72).

1.5.2 Indirect assimilation of ferrous iron:

1.5.2.1 Hemophores and Heme oxygenases: Heme is an iron containing porphyrin molecule. Heme serves in a multitude of cellular functions such as respiration, oxygen

transport, detoxification and signal transduction (80). However, free heme is toxic to cells. Therefore, heme is primarily coupled with carrier proteins as hemoglogin, haptoglobin, hemopexin and albumin.

Heme serves as an iron source for several bacterial species (11, 16, 29). As mentioned above, bacterial species such as *Neisseria meningitidis* and *Yersinia enterocolitica* can directly come in contact with hemoproteins and via receptor mediated interaction can acquire iron. Others such as *Hemophilus influenzae,* and *Pseudomonas aeruginosa* can secrete specialized extracellular proteins called Hemophores. *H. influenzae* secretes HxuA, a 100kDa protein, that acquires iron from hemopexin (19). A pore forming accessory protein, HxuB, secretes HxuA. HxuA binds heme-loaded hemopexin and the complex is delivered to an outer membrane specific receptor, HxuC (18, 20). Hemophores secreted by *P. aeruginosa* extract heme from hemoglobin and, present it to outer membrane receptors for internalization. The proteins involved in the secretory apparatus, the hemophore, and the outer membrane receptor is assembled as an operon, which is repressed by FuR in the presence of iron (39). The Hemophores produced by *P. aeruginosa* called Has A (heme acquisition system) are secreted by ABC transporters constituted by three envelope proteins – an inner membrane ATPase, a membrane fusion protein and an outer membrane component that belongs to the TolC family (146).

Heme oxygenases (HOs) are involved in heme catabolism, iron utilization and cellular signaling (110). HOs are well characterized in eukaryotic systems. The first HO was identified as a mammalian enzyme that cleaves heme to release bilivirdin along with

free iron and carbon monoxide (154). However, HOs are also widespread among prokaryotes and bacterial pathogens can acquire iron by oxidative cleavage of heme. In cyanobacteria HOs are also involved in the phytobilin synthesis, which are chemophore precursors used by the light-harvesting phycobiliproteins and the photoreceptor phytochrome (155). However, in others such as *Corynebacterium diptheriae, Neisseria meningitidis*, and *Pseudomonas aeruginosa* HO, degrades heme to bilivirdin, iron and carbon monoxide in the bacterial cytoplasm (120, 121, 123). Heme degraders made by *Staphylococcus aureus* and *Bacillus anthracis* do not share structural homology to canonical HO, however, they still retain the capability to degrade heme (127).

While mycobacteria carry the machinery for a biosynthetic pathway of heme, they do not produce hemophores (67). However, recently the crystal structure of a heme degrader called MhuD (mycobacterial heme utilization, degrader) from *M. tuberculosis* has been reported (17). MhuD shares sequence similarity with IsdG and IsdI heme degraders of *S. aureus.* MhuD binds two molecules of heme per monomer. MhuD can serve as a potential drug target because MhuD is structurally different from eukaryotic HO, and is intricately involved in iron recycling, and iron is essential for mycobacterial survival inside the host.

1.5.2.2 <u>Siderophores:</u> Siderophores are small molecular weight iron-chelating molecules produced by many microorganisms (30, 31). The iron binding efficiency of siderophores is sufficiently strong to remove iron coupled with host proteins such as ferritin, transferrin and lactoferrin (158). Siderophore biosynthesis is derepressed when cells are

grown under iron deficiency. Siderophore production is not intricately involved with pathogenicity as non-pathogenic bacteria also produce siderophores. Alcaligin (AG) is the first ferric dihydroxamate siderophore identified in the nonpathogenic microbe, *Alcaligenes denitrificans (56).* However, siderophore production by a pathogen may confer virulence. A siderophore deficient mutant of *M. tuberculosis* has decreased ability to survive inside macrophages (26).

Siderophores are assembled by nonribosomal, cytoplasmic peptide synthases resembling antibiotic synthesis machinery (24, 30). Siderophores are exported into the environment via proton-motive force (pmf) dependent membrane efflux pumps (35). Bacteria can also acquire iron from siderophores produced by other microbes living in the vicinity (78). Iron laden siderophores are recognized at the cell surface by the specific outer membrane receptors in Gram-negative bacteria and by membrane anchored proteins in Gram-positive bacteria (100). However, in certain cases iron can be shuttled between several different siderophores prior to uptake (25).

1.5.2.2.1 <u>Enterobactins:</u> Enterobactins are siderophores secreted by *Salmonella enterica* serovar Typhimurium and *Escherichia coli (86).* Enterobactin genes are also found in other pathogens such as *Salmonella, Klebsiella, Shigella* and *Pseudomonas* species. *E. coli* produces several structurally different siderophores such as enterobactin, aerobactin, yersiniabactin and salmochelins (42). *Vibrio* species produce vibriobactin and anguibactin. The initial stage of enterobactin synthesis requires the product of three genes: *entC,* which encodes an isochorismate synthase; *entB,* which encodes 2,3-dihydro-

2,3-dihydroxybenzoate synthase, and *entA*, which encodes 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (37, 38). The second stage deals with the synthesis of one molecule of enterobactin from three molecules each of 2,3-dihydroxybenzoic acid and L-serine. Products of the genes *entD, entE,* and *entF* and an assembly activity encoded in the carboxy terminus of *entB* catalyze this step. The cytosolic proteins EntE, EntF, and EntB, which are *E. coli* enzymes necessary for the final stage of enterobactin synthesis, are released by osmotic shock. Enterobactins may be secreted directly from its site of synthesis through the envelope to the external milieu.

Siderophore synthesis and the transport apparatus are organized on a 22-kb gene cluster (136). Transport of ferric enterobactin into the cell cytosol requires the products of five genes. An outer membrane receptor for ferric enterobactin is encoded by *fepA.* The gene product of *fes* is required for cytosolic release of iron from enterobactin. Gene products of *fepB, fepC, fepD, fepE,* and *fepG* are required for uptake of ferric enterobactin through the periplasm and the cytoplasmic membrane.

Because iron acquisition is important, it is not surprising that several bacterial species produce more than one type of siderophore and employ more than one iron transport systems. For e.g.: *E. coli* has as many as six ferric iron transport systems described encoded by *fec, fep, fhu, iro, aerobactin* and *fheE.* Furthermore, a novel iron transport system called *fit* was identified by in vivo expression technology (89).

1.5.2.2.2 <u>Mycobactins:</u> Mycobacteria are known to produce three types of siderophores – mycobactins, carboxymycobactins and exochelins (99, 158, 163, 165). Mycobactin is

found in all pathogenic and non-pathogenic mycobacteria except *M. vaccae* and *M. avium* subsp. *paratuberculosis*. However, the *M. avium* subsp. *paratuberculosis* (MAP) genome contains the promoter for the mycobactin biosynthetic operon, which is active (58). Mycobactin is synthesized along with two polyketide synthases (MbtC and MbtD), three peptide synthases (MbtB, MbtE, and MbtF) and isochorismate synthase (MbtI), which provides salicylic acid (99). Mycobactins are lipid soluble. In order to acquire iron from extracellular environments, *Mycobacterium tuberculosis* also produces water-soluble siderophores called carboxymycobactins that have shorter acyl chains ending with a carboxylic acid group. The biosynthesis of carboxymycobactins is similar to that of mycobactins (107). The uptake of ferric carboxymycobactins is facilitated via an ABC transporter encoded by *irtA* and *irtB (116).* Whether *M. avium* subsp. *paratuberculosis* produces carboxymycobactins is currently unknown. Additionally, the non-pathogenic mycobacterium *M. smegmatis* produces water-soluble siderophores called exochelins (165). A non-ribosomal peptide synthase complex encoded by *fxB* and *fxC* produces the exochelins. Additional genes, *fxuA, fxuB,* and *fxuC,* encode proteins needed for ferric exochelin uptake while ExiT plays a role in export of exochelins.

In order to adapt to an iron deficiency state, bacteria have developed a series of responses. Besides the synthesis of siderophores and/or hemophores and iron uptake and transport systems, bacteria also express a plethora of virulence genes that remain silent under iron sufficient growth. For e.g.: Pathogenic bacteria such as *C. diphtheriae, S. dysenteriae, P. aeruginosa,* and *N. meningtidis* produce toxins in response to iron

limitation within the host (23, 98). Most of these genes induced under iron-deprived conditions are under the tight control of transcriptional factors.

1.6 Transcriptional control of intracellular iron homeostasis:

Iron is a critical factor in determining the ability of a pathogen to establish successful infection. To combat pathogens, the host presents either an iron deficient environment or uses excess iron to generate reactive oxygen species. Regulatory proteins control iron homeostasis in bacteria (22). The ferric uptake regulator (Fur) and Diphtheria toxin repressor (DtxR) are very well characterized as global iron regulators.

1.6.1 Fur as a global regulator of gene expression: Fur is a 17kDa protein produced by *E. coli (50).* Fur also controls iron dependent genes in several Gram-negative bacteria such as *P. aeruginosa* and Gram-positive bacteria including *B. subtilis (52, 135, 139).* There are two Fur orthologs (FurA and FurB) in the *M. tuberculosis* genome (111). FurB is involved in zinc homeostasis while FurA regulates expression of catalase (*katG*) (77). However, the *M. avium* subsp. *paratuberculosis* genome encodes three Fur-like proteins, one of which is located on a pathogenicity island (131). The role of Fur in *M. avium* subsp. *paratuberculosis* is currently unknown.

Under iron sufficient conditions, Fe^{2+} is delivered to Fur proteins by the ferri siderophore reductase system coupled to either 2,3-dihydroxybenzoic acid or salicylic acid (50). The Fur-Fe^{2+} complex then binds to a specific 19 base pair palindrome sequence on the promoter called the “Fur box” thereby regulating expression of over 30

genes in *P. aeruginosa* (87). Fur represses genes coding for the synthesis of siderophores (pyoverdin, pyochelin, enterobactin, ferrioxamines), heme uptake and other systems for iron uptake, an alternative sigma factor, regulators of other genes, regulator for cell division, and other functions. However, genes derepressed under iron rich conditions include, bacterioferritin synthesis, oxidative stress response, catalase, peroxidase and superoxide dismutase. Due to its ability to control expression of genes involved in wide variety of cellular functions, Fur is considered a global regulator. The *fur* gene is essential for some bacteria such as *P. aeruginosa, P. putida,* and *N. gonorrhoeae,* in contrast to its dispensability in *E. coli, Vibrio* spp., and *Y. pestis (141, 142).*

Under iron limiting conditions, cells prioritize iron by reserving it for the synthesis of essential proteins such as heme, cytochromes and ferric enzymes; therefore, iron is unavailable for Fur to bind (117). The low iron affinity of Fur and its ability to attain a non-DNA binding form has a fitness advantage, as it would ensure that genes required for iron acquisition remain derepressed.

1.6.2 <u>DtxR as a global regulator of gene expression:</u> Several Gram-positive bacteria produce a protein that functions similarly to Fur but is structurally distinct (14, 34). Similar to Fur, DtxR (diphtheria toxin repressor) of *C. diphtheriae* is activated in the presence of Fe^{2+}. It binds the operator sequences on the promoter of the *tox* (Diphtheria toxin) gene as a dimer activated by iron. DtxR-like proteins are found in other Gram-positive bacteria such as *Streptomyces* spp., *Brevibacterium* spp., and *Mycobacterium* spp., which also produce a Fur-like protein (45, 54, 122).

DtxR regulon analysis of *C. glutamicum,* an industrial microorganism predominantly used for large-scale production of amino acid L-glutamate, resulted in novel findings (151). As expected, DtxR of *C. glutamicum* regulates expression of genes involved in iron transport and siderophore interacting proteins, iron utilization, storage and protection, citric acid cycle enzymes, secreted proteins and other regulatory proteins. Interestingly, DtxR of *C. glutamicum* also represses the expression of a transcriptional factor called RipA (regulator of iron proteins) in the presence of iron (152). Under iron-limitation, DtxR no longer binds the *ripA* promoter and RipA is synthesized. Newly made RipA under iron-limiting conditions is a repressor of iron utilization proteins such as aconitase, succinate dehydrogenase and catalase. This regulatory cascade helps cells to prioritize iron for essential proteins under iron-limitation.

In sum, both Fur and DtxR recognize a unique sequence termed "*fur* box" or "*ideR* box" respectively on the promoter regions and regulate downstream gene expression. Following activation in the presence of iron, Fur and DtxR repress iron acquisition genes while activating iron storage genes. In addition to maintaining iron homeostasis, these regulators are also involved in oxidative stress response and other virulence factors such as toxin production. For example, in *Corynebacterium diphtheriae,* toxin production is a function of the amount of iron and is regulated by DtxR. These studies suggest that prokaryotes have developed complex regulatory mechanisms that can precisely detect iron inadequacy or iron mediated intoxication.

1.7 Post-transcriptional control of intracellular iron homeostasis:

Posttranscriptional regulation of maintaining the intracellular iron pool is emerging as a common theme in bacteria (109). *RhyB*, a small RNA has been shown to regulate gene expression in an iron dependent fashion (79). When intra cellular levels of iron are low, *RhyB* represses transcription of non-essential iron using proteins and favors transcription of essential proteins such as ribonucleotide reductase. Apart from Fur controlling expression of *RhyB,* evidence suggests that *RhyB* is sufficient to regulate intracellular iron concentrations. *RhyB* acts by complementary pairing to its target *mRNA* thus making target *mRNA* vulnerable to degradation. In *Shigella flexneri, RhyB* also plays a role in acid resistance where as in *Vibrio cholera* it plays a role in biofilm formation and chemotaxis (41, 92). Thus *RhyB* has pleiotropic effects on bacterial gene expression in response to host. Two small RNAs *prrF1* and *prrF2* of *Pseudomonas aeruginosa* control expression of almost 60 genes that play a role in iron metabolism (138). More recently, in *Bacillus subtilis* a small RNA called *FsrA* was identified (36). *FsrA* in iron limiting conditions, repressed transcription of non-essential iron using proteins thus sparing iron for essential proteins such as those involved in citric acid cycle. *However,* in *Saccharomyces cerevisiae* a similar goal is achieved via a zinc-finger RNA binding protein, Cth2 (132). In *Corynebacterium glutamicum* iron sparing response is regulated by DtxR regulated protein called RipA (152).

Although small RNAs appear to exist in mycobacteria, the existence of iron dependent posttranscriptional regulation mediated via small RNAs and/or regulatory proteins is currently unknown.

1.8 Iron regulation in mycobacteria and the role of Iron dependent regulator:

The amount of iron required for optimal growth of mycobacteria for *in-vitro* cultures has been estimated to be about 7 to 64μg per gram of cell mass (106). In order to meet required iron concentrations, mycobacteria produce siderophores called mycobactins (25). In *Mycobacterium tuberculosis* (*MTB*) two genetic loci have been identified that are involved in siderophore synthesis (66). Pathogenic mycobacteria accumulate large amounts of iron (1000 to 2000 μM/L) inside a macrophage, as early as 24-hr post infection relative to mycobactin deficient mycobacteria (289 μM/L) and non-pathogenic mycobacteria (41.5μM/L) (143, 144). Iron overload in phagosomes is generally reversed when macrophages are activated with IFN-gamma suggesting that pathogenic mycobacteria undergo both iron toxicity and deprivation within the phagosome environment. Therefore, mycobacteria utilize a delicate regulatory mechanism to acquire, store or prevent accumulation of iron. This is accomplished by the iron dependent regulator (IdeR) (112).

IdeR is a member of DtxR family of regulatory elements present in MTB and plays a role in global iron regulation. Comparative evaluation of mycobacterial genomes has revealed that IdeR is present in all of the sequenced pathogenic and non-pathogenic mycobacteria (162). Structural and functional roles of IdeR are extensively studied in

Mycobacterium tuberculosis (MTB) (95, 156, 157). Upon activation in the presence of iron, IdeR recognizes a 19-bp promoter sequence called the "**iron box**" and regulates transcription of genes involved in iron acquisition, iron storage and oxidative stress (40). *ideR* is an essential gene in MTB *(113).* Inactivation of *ideR* is possible only in the presence of a second suppressor mutation. When cellular levels of iron are high, IdeR represses mycobactin synthesis genes and de-represses genes involved in iron storage and genes necessary to combat oxidative stress (113). Thus, IdeR is a global iron regulator in MTB iron homeostasis (Figure 1).

1.9 Iron acquisition by mycobacteria inside macrophages:

Pathogenic mycobacteria including MTB and MAP persistently infect and survive inside macrophages amid nutrient deprivation and oxidative stress. Iron is essential for the survival of mycobacteria (108). Thus the mycobacterial phagosome presents a barrier to pilfer iron from the host. Therefore in order to survive inside phagocytes, pathogenic mycobacteria need to acquire iron from macrophages. Molecular mechanism of mycobactin mediated iron acquisition by MTB inside the macrophage has been proposed recently (76). It is suggested that following internalization by macrophages, mycobacteria produce mycobactins which are distributed into inter- and intra-cellular compartments. These mycobactins mobilize iron into the mycobacterial phagosome. This further enhances ferric-transferrin mediated transport of iron into the intracellular iron storage compartment of the macrophage. A mycobactin deficient MTB strain demonstrated reduced colonization in mice (26). Taken together, it is clear that iron regulation is

essential for survival of pathogenic mycobacteria. In order to acquire this physiologically important metal inside the host, pathogenic mycobacteria employ mycobactin mediated iron acquisition. IdeR controls a network of genes involved in maintaining iron homeostasis (iron acquisition, iron transport and iron storage).

Mycobacterium avium subsp. *paratuberculosis* (MAP) is very well known for its unique iron requirements for in vitro growth (82, 129). Mycobactin dependency in laboratory based culture systems is now a conventional wisdom. In MAP, there are two genomic islands with significant homology to siderophore biosynthesis (73, 131). MAP also encodes genes for IdeR and Fur. Furthermore, one of the putative siderophore biosynthetic operon and *fur* are located on a MAP specific pathogenicity island (131). An extracellular ferric reductase characterized from MAP has been shown to play a possible role in transportation of ferric iron inside the cell (55). MAP regulates expression of genes involved in iron acquisition inside the macrophages (58, 166). These findings suggest that MAP employs a complex regulatory network of genes that are deployed to maintain iron homeostasis (Figure 2).

Figure 1: Iron transport in mycobacteria

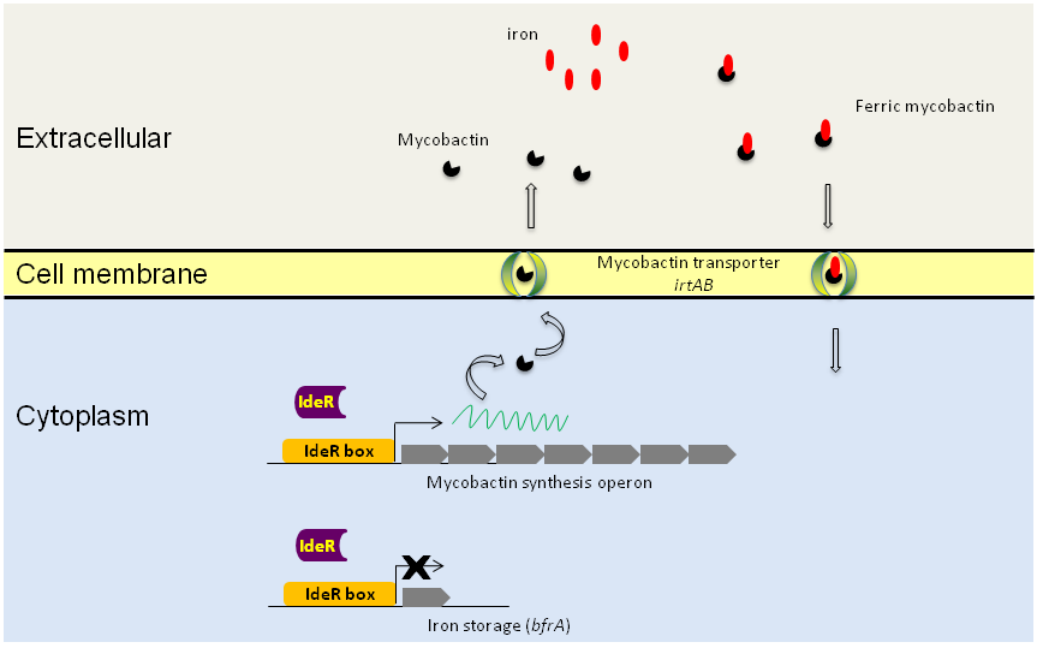

Figure 1: Under iron limiting conditions, IdeR derepresses the synthesis of mycobactins which are transported into the extracellular environment via ABC type transporter called irtAB. Iron loaded mycobactin (ferric mycobactin) is again transported back into the intracellular environment by irtAB.

Figure 2: Paradigm for regulation of iron utilization proteins

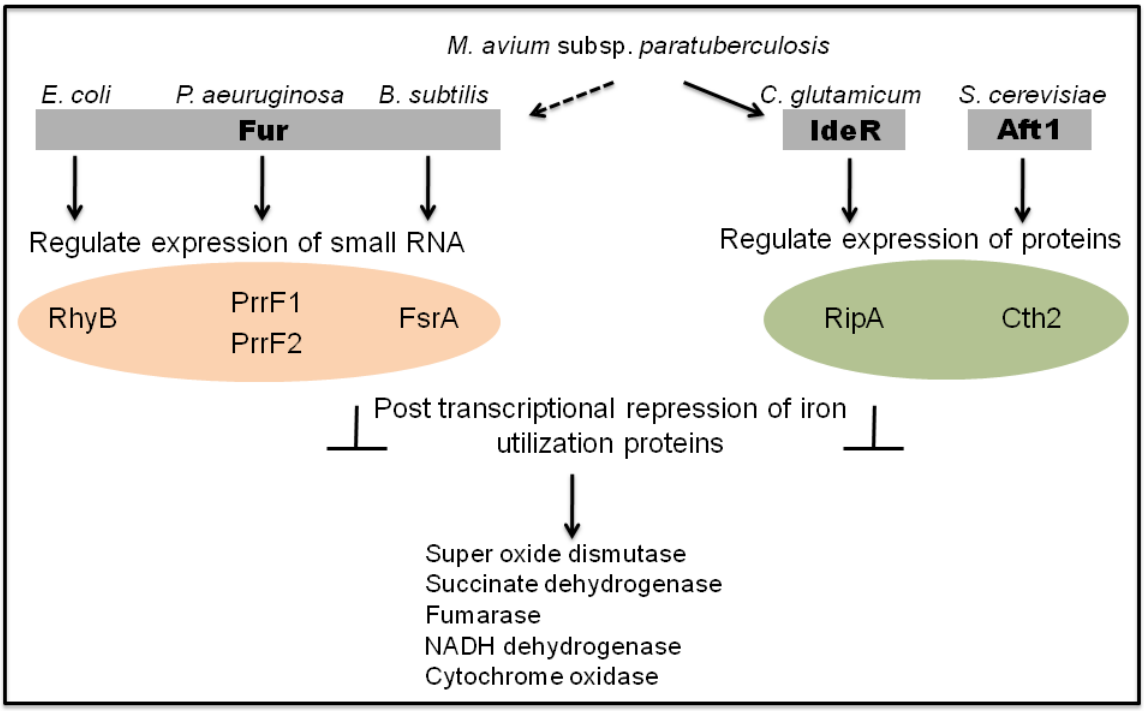

Figure 2: Under iron limiting conditions transcriptional regulators (Fur, IdeR or Aft1) derepress the expression of either small RNAs or regulatory proteins that repress the expression of other iron utilization proteins. MAP IdeR regulation and posttranscriptional repression of iron utilization proteins is known, however, the role of Fur in MAP needs to be tested.

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CHAPTER 3: IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF IRON DEPENDENT REGULATOR (IDER) OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS**

Mycobacterium avium subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle and sheep, has unique iron requirements in that it is mycobactin dependent for cultivation *in-vitro*. The iron dependent regulator (IdeR) is a well-characterized global regulator responsible for maintaining iron homeostasis in *Mycobacterium tuberculosis* (MTB). We identified an orthologous segment in MAP genome, MAP2827, with >93% amino acid identity to MTB IdeR. Electrophoretic mobility shift assays and DNase protection assays confirmed that MAP2827 protein binds the 19 base pair consensus motif (iron box) on the MAP genome. Resequencing MAP2827 from multiple isolates revealed a non-synonymous change (R91G) exclusive to sheep strains. Reporter gene assays and quantitative real time RT-PCR assays in two diverse MAP strains and in an *ideR* deletion mutant of *M. smegmatis* (mc^2155) suggested that both sheep MAP IdeR (sIdeR) and cattle MAP IdeR (cIdeR) repressed *mbtB* transcription at high iron concentration and relieved repression at low iron concentration. On the other hand, *bfrA* (an iron storage gene) was upregulated by cIdeR when presented with MTB or cattle MAP *bfrA* promoter and was downregulated by sIdeR in the presence of MTB or sheep or cattle MAP *bfrA* promoters, at high iron concentration. The differential iron regulatory mechanisms between IdeR regulated genes across strains may contribute to the differential growth or pathogenic characteristics of sheep and cattle

MAP strains. Taken together, our study provides a possible reason for mycobactin dependency and suggests strong implications in the differential iron acquisition and storage mechanisms in MAP.

INTRODUCTION

Iron is a cofactor in several enzymatic reactions due to its wide redox potential (2, 21). Iron plays a central role in enzymatic reactions involved in electron transport, nucleic acid synthesis and oxidative stress defense. Therefore, iron is critical for most living systems. The iron dependent regulator (IdeR) of MTB has been shown to regulate a repertoire of genes in response to iron concentration (16). Comparative evaluation of mycobacterial genomes has revealed that *ideR* is present in all of the sequenced mycobacteria (26). Structural and functional roles of IdeR have been extensively studied in MTB (13, 15, 23, 24). In the presence of iron, IdeR binds to a 19-bp promoter sequence called the "iron box" and represses the transcription of genes involved in iron acquisition while activating iron storage genes. Inactivation of *ideR* is possible only in the presence of a second suppressor mutation suggesting that *ideR* is an essential gene in MTB (16). This rare mutant of *ideR* showed deregulated siderophore synthesis and increased sensitivity to oxidative stress. Thus it is clear that iron concentration induced gene regulation is essential for the survival of pathogenic mycobacteria.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is very well known for its unique iron requirements for *in-vitro* growth. Mycobactin dependency in laboratory

based culture systems is now well-established (22). We recently demonstrated that despite mycobactin dependency *in-vitro*, MAP upregulates mycobactin (*mbt*) synthesis genes inside bovine macrophages (27). Similarly proteomic (MAP grown in iron sufficient and iron limited conditions) analysis has also revealed that MAP regulates expression of iron storage (*bfrA*) gene (Janagama and Sreevatsan; submitted). These findings suggest that MAP employs a network of genes that are deployed to maintain iron homeostasis. IdeR, a transcriptional factor monitors global iron regulation in MTB. Currently, there is a lack of understanding of iron regulation in MAP. In the current study, we characterized the IdeR of MAP and its functional role in iron dependent gene regulation.

MATERIALS AND METHODS

Bacterial strains, DNA manipulations and protein expression: *Mycobacterium avium* subsp. *paratuberculosis* strains were grown in Middle brook 7H9 supplemented with OADC enrichment medium and mycobactin J. *M. smegmatis* was grown in Luria Bertani (LB) medium. Antibiotics (kanamycin (20 µg/ml), hygromycin (100 µg/ml), streptomycin (20 µg/ml) and spectinomycin (75 µg/ml)) were added when necessary. *E. coli* BL21 (DE3) competent cells (Novagen, Madison, WI) and *E. coli* TOP10F competent cells (Invitrogen) were grown in LB medium. Minimal medium was prepared as described previously (16). DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA). The predicted open reading frame of MAP2827 from

MAP K-10 genome was amplified using primers (2827NF : 5’ GGAATTCCATATGATGAACGACCTGGTTGACACC 3’ and 2827BR: 5’ CGCGGATCCTCAGACCTTTTCGACCTTGA 3’), which carried NdeI and BamHI restriction sites (highlighted) at the 5'end and cloned into predigested (NdeI and BamHI) pET-16b vector (Novagen, WI). Proper insertion and orientation of MAP2827 into pET-16b vector was verified by sequencing. BL21 (DE3) *E. coli* competent cells (Novagen, Madison, WI) were transformed with pET-16b vector carrying MAP2827 and induced with 1mM isopropyl β-D-1thiogalactopyranoside (IPTG) for 4 h to over express MAP2827. The expressed MAP2827 with N terminal his-tag was purified using Ni-NTA columns (Qiagen, Valencia, CA). Recombinant MAP2827 was also obtained as maltose binding protein (MBP) fusion protein (1).

Computational prediction of IdeR regulated genes: MTB IdeR regulates gene expression by binding to a 19bp consensus sequence termed iron box in the promoter regions (6). Prokaryotic database of gene regulator (PRODORIC) is a knowledge base that encompasses molecular networks of prokaryotes such as transcriptional regulation (www.prodoric.de) (11). Virtual footprint, a software tool of PRODORIC specializes in identifying the transcriptional factor binding sites in a bacterial genome. Using this software option, iron box sequence (TWAGGTWAGSCTWACCTWA; where W = A/T and S = G/C) was queried on the MAP genome (7) to retrieve similar sequences in the intergenic regions. Searches were allowed for five mismatches in the consensus sequence

and were limited to -300 to +100 bases of each predicted open reading frame as previously reported (6).

Electrophoretic mobility shift assay (EMSA): Physical binding of Cattle MAP2827 to the promoter sequences of cattle *mbtB* and *bfrA* was carried out by EMSA. Promoter sequences containing the putative iron box of MAP *bfrA* or *mbtB* were amplified using 5' biotin labeled primers via PCR. Purified amplification products were used in DNA protein interactions. Binding reactions (6) were performed for 30 min in the presence or absence of 200 µM $NiSO_4$ containing 20fm of DNA and 250ng of the protein and the complexes were resolved in a 10% non-denaturing polyacrylamide gel. Following gel electrophoresis, the complexes were transferred onto a nylon membrane and detected using chemiluminiscence based nucleic acid detection (Pierce, Rockford, IL).

DNase protection assay: DNase foot printing assay was performed to map the physical binding region of MAP2827 on the putative promoter sequence as described previously (8, 28). Cattle MAP *mbtB* or *bfrA* promoters carrying predicted iron box sequence were cloned into a plasmid pSM128 (detailed below). A 5' FAM labeled forward primer (which had the priming sites 100bp upstream of the cloned segment) and a 5' HEX labeled reverse primer (which had the priming sites 100bp downstream of the cloned segment) were used to amplify MAP promoters from pSM128. Binding reactions of MAP2827 to the probe was performed similar to those described for EMSA. Following DNA protein interaction, complexes were digested partially with DNaseI. A parallel

partial DNaseI digestion of the DNA probe alone was performed. Digested DNA and DNA protein complexes were purified individually in order to remove proteins and other salts contained in the reaction buffer. Purified fragments were resolved in a fragment analyzer (Applied Biosystems 3130xl, Foster City, CA) and chromatograms were analyzed using GeneMapper software (Applied Biosystems, Foster City, CA).

Reporter gene assays: Functional activity of *mbtB* and *bfrA* promoters was assessed using a cattle MAP strain (MAP1018) and a *M. smegmatis* ideR null mutant, SM3 (3). Predicted MAP promoter sequences containing the iron box were amplified using primers carrying ScaI restriction sites. Amplified products were restriction digested with ScaI and ligated into a predigested (ScaI) promoterless integrative plasmid, pSM128 (4). Proper orientation of sequences into the plasmid was verified by sequencing. MAP1018 was transformed with pSM128 carrying MAP mbtB or *bfrA* promoters. Open reading frames MAP2827 from MAP K-10 (cattle strain) or MAP 7565 (sheep strain) were amplified via PCR using primers that carried restriction sites for BamHI and HindIII. Amplified products were double digested with BamHI and HindIII and ligated into a pre digested (BamHI and HindIII) expression plasmid pSM417. Proper orientation and ligation of MAP2827 into pSM417 was verified by sequencing. SM3 was transformed both with pSM128 carrying MAP promoter sequences and pSM417 carrying MAP2827. The recombinant MAP1018 (carrying MAP *mbtB* and *bfrA* promoter lacZ fusions) and *M. smegmatis* (carrying MAP mbtB or bfrA promoter lacZ fusions and MAP2827) strains were cultured in minimal medium supplemented with 50 µM (high iron) or 1 µM of

$FeCl_3$ (low iron) and analyzed for β galactosidase activity (14) per manufacturer's recommendations (Promega, Madison, WI ; cat#E2000). Iron concentrations were selected based on the fact that a concentration above 25 µM is sufficient to activate the repressor activity of IdeR (Rodriguez *et al*. unpublished).

Real time Q-RT PCR assays: Transcription of *mbtB* and *bfrA* was measured using real time Q- RT PCR. MAP 1018 (cattle strain) and MAP 7565 (sheep strain) was grown to mid logarithmic growth phase (OD_{600} = 1.0) in MB7H9 medium containing OADC and mycobactin J. Bacterial cultures were washed five times in 1X PBS, re-suspended in the minimal medium containing no iron or iron at 50 µM concentration and allowed for gene expression for 3 hours. RNA isolations were performed as described earlier (27). RNA was treated with DNaseI (Ambion, Austin, TX) and Q-RT PCR was performed using QuantiFast SYBR Green mix (Qiagen, Valencia, CA) and gene specific primers in a Lighcycler 480 (Roche, Indianapolis, IN). Gene expression values of *mbtB* and *bfrA* was normalized to 16s rRNA and reported as fold change (Low iron/High iron).

Intracellular and in-vitro MAP gene expression profiling: Human primary CD14 positive monocytes were obtained from Lonza (Walkersville, MD). Monocytes were differentiated into macrophages by the addition of recombinant human granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). Following differentiation for three days in the Teflon wells (Savillex Corporation, Minnetonka, MN), 2 x 10^6 macrophages were seeded into 25cm^2 tissue culture flasks. Macrophages

were infected with cattle or human MAP strain at a multiplicity of infection of 20. RNA isolation and cDNA synthesis were performed as described earlier with the following modifications (25, 27). Briefly, first strand cDNA was synthesized from total RNA isolated from infected macrophages or MAP grown in broth cultures using primers that carried defined 5′ end and a random nonamer at 3′ end. MAP targets specifically expressed inside macrophages were obtained via hybridizations to biotin labeled MAP genomic DNA. Following second strand cDNA synthesis and PCR amplification, MAP cDNA obtained either from macrophages or broth cultures was individually hybridized onto MAP K-10 microarrays. MAP genomic DNA (gDNA) was used as a reference in each hybridization (5). Array normalizations and data analysis were performed as described (12). Following normalization of gDNA and cDNA signals, fluorescence intensities were $\log_2$ transformed and reported as fold-change. Genomic DNA based normalization of expression data has been described before (17).

Statistical analysis: All experiments were independently repeated at least three times in triplicate. Means of Miller Units from reporter gene assays were compared across strains and treatments using a multiple range t-test with Bonferroni's correction.

RESULTS

MAP2827 shows sequence specific physical binding to the promoter regions of mbtB and bfrA: The annotated version of MAP IdeR (MAP2827c) did not share aminoacid

identities to other mycobacterial IdeR proteins. However, DNA sequence alignment of MAP2827c and MTB IdeR revealed 86% nucleic acid similarity. This suggested that MAP IdeR may be transcribed in a different open reading frame in the same genomic location thus revealing MAP2827 as a possible open reading frame encoding MAP IdeR.

Computational predictions identified 24 genes that may be regulated by IdeR (Table S1). We focused our analyses on proteins that serve 2 distinct functions - an iron acquisition protein, MbtB, and an iron storage protein, BfrA. We first tested the ability of MAP IdeR to bind to the predicted promoter regions using electrophoretic mobility shift assays (EMSA). Our results show that IdeR bound to the promoters of *mbtB* and *bfrA* in presence of Ni^{+2} (Figure 3). A competition for the binding sites on IdeR using excess unlabeled promoter DNA abrogated the gel shift. Furthermore, there was no binding of IdeR to a divergent probe that carried no iron box sequence. The DNA sequence bound by IdeR was identified by DNase protection assays using fluorescently labeled intergenic regions of *mbtB* or *bfrA* containing the suspected iron box sequence. Results show that IdeR recognized and protected the iron box sequence on the MAP *mbtB* and *bfrA* promoters (Figure 4). These results demonstrate that MAP IdeR is a metal-dependent DNA binding protein that interacts with a specific sequence in the promoter regions of iron regulated genes and is likely to function as a transcriptional regulator in MAP.

Sequence analysis of the MAP ideR open reading frame and promoter regions of putative IdeR regulated genes reveals polymorphisms between sheep and cattle MAP strains: To establish that MAP2827/*ideR* was conserved across all genotypes of

MAP, ideR and the *mbtB* and *bfrA* promoters of putative IdeR regulated genes from cattle, human, bison, and sheep MAP isolates were sequenced. Data showed that ideR was conserved across all cattle (1018, K-10, 9123, 9142, 9245), bison (7560), and human (M4, M5, M6) isolates studied, but all sheep isolates (7565, 467, 394) showed a single nucleotide polymorphism that led to an amino acid change at residue 91 (R91G) (FigureS1). Similarly sequence analysis of the *mbtB* and *bfrA* promoter regions of IdeR transcribed genes identified polymorphisms exclusively in sheep MAP strains (Figure 5). Of the two iron boxes present within the *bfrA* promoter, the iron box distal to bfrA start site in sheep MAP isolates is deleted. Similarly, the *mbtB* promoter of sheep MAP isolates carried two single nucleotide polymorphisms (C-87G and G-71 A relative to the ATG site of *mbtA*) compared to cattle MAP isolates. C to G polymorphism is present within whereas G to A polymorphism is present outside the iron box.

Functional assays reveal divergent control of promoters by sheep and cattle MAP IdeR: Transcriptional regulation of IdeR derived from cattle (cIdeR) and sheep (sIdeR) MAP strains were studied using promoter fusions to *lacZ* in an *ideR* deletion mutant of *M. smegmatis* (SM3) and in intact cattle and sheep strains of MAP. Controls used were dual empty vector (SM3 transformed with pSM128 and pSM417 without inserts), promoter alone control (SM3 transformed with pSM128 carrying promoter without iron box sequence), iron box control (SM3 transformed with pSM128 carrying promoter with iron box sequence), *ideR* alone control (SM3 transformed with pSM417 carrying *ideR* of cattle or sheep MAP variant) grown in high or low iron medium.

First, control experiments in an *ideR* deleted *M. smegmatis* (SM3) showed that cIdeR or sIdeR repressed transcription of MTB *mbtB* under high iron conditions similar to MTB IdeR (Figure 6A). Since sequence analysis of ideR and the promoter regions of IdeR regulated genes revealed polymorphisms between cattle and sheep MAP strains, we tested structure function associations by swapping *ideR* and promoters between cattle and sheep MAP variants and measured transcription of the reporter gene in SM3. Results suggested that irrespective of polymorphisms in the promoter segments, *mbtB* transcription was repressed under iron rich conditions by c and sIdeR (Figure 6B and 6C). These findings were statistically significant at $P < 0.05$.

We then compared the activities of MTB (control), cIdeR, and sIdeR on *bfrA* promoter binding and subsequent transcription. MTB IdeR and cIdeR showed identical activities on binding to cattle MAP or MTB *bfrA* promoters under iron rich conditions (Figure 7A, 7B). In contrast, IdeRs from MTB, sheep and cattle strains of MAP downregulated transcription in iron excess growth medium in the presence of sheep *bfrA* promoter (Figure 7C).

Second, these effects were studied in a native MAP background - we attempted to create an ideR deletion mutant of MAP and failed. Therefore, to address if the promoters are active in MAP genetic background we transformed cattle and sheep MAP strains with *bfrA* or *mbtB* promoters and measured reporter activity. Results suggested that both *mbtB* and *bfrA* promoters are active in MAP (Figure 8). In the presence of excess iron, both c and sIdeR repressed *mbtB* transcription where as only sIdeR repressed transcription via *bfrA* promoter.

Since reporter gene assays suggested differential promoter activities and downstream gene expression between cattle and sheep MAP strains, we measured transcription of *mbtB* and *bfrA* in cattle and sheep MAP strains using Q-RT PCR under high and low iron concentrations. Consistent with reporter gene assay data in SM3 and MAP background, results showed that *mbtB* was derepressed under low iron growth conditions by cattle and sheep MAP strains whereas *bfrA* transcription was derepressed only in sheep MAP strain (Table 1).

Next, we performed microarray based gene expression analysis on a cattle strain to confirm both functional assay data and computational predictions. Several of the predicted genes were transcribed inside infected macrophages (iron depleted environment) or in nutrient rich broth cultures (Table 2). As expected *mbtA* was downregulated whereas *bfrA* was upregulated in the nutrient rich broth cultures in a cattle MAP strain. *mbtE* was upregulated inside human macrophages. Expression data of *bfrA* in broth cultures (iron rich environment) and *mbtE* inside macrophages (iron deficient) clearly suggests that MAP regulates expression of these genes in an iron dependent fashion.

DISCUSSION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is unique in its iron requirements owing to its mycobactin dependency in vitro. However, to date there is no comprehensive understanding either of iron metabolic pathways, in general, or the regulation of

mycobactin operon in MAP. In *M. tuberculosis* (MTB), the iron dependent regulator, IdeR is the principal regulator of iron metabolism (15). We here report that MAP2827 carries IdeR function and regulates *mbtB* and *bfrA* gene expression. Selection of *mbtB* and *bfrA* is based on their immediate roles in iron acquisition and iron storage, respectively.

IdeR controls expression of genes involved in iron acquisition and iron storage via iron box sequence recognition and binding. Computational screening of the MAP genome for iron box motifs identified 24 genes associated with diverse functions in iron metabolism. Transcriptional analysis confirmed expression of a subset of these genes in MAP upon macrophage infection (iron restricted conditions) and/or broth cultures (iron rich media). Expression data of *bfrA* in broth cultures (iron rich environment) and *mbtE* inside macrophages (iron deficient) clearly suggests that MAP regulates expression of these genes in an iron dependent fashion. *mbtE* is present in the MAP genome as a part of mycobactin synthesis operon where the iron box is located between *mbtB* and *mbtA*. These findings suggest that MAP IdeR regulates at least some of the genes computationally predicted in the IdeR regulon in this study. Genome mining to identify an IdeR homolog in the MAP genome revealed that the complementary sequence of the annotated ideR shared substantial amino acid similarities with the IdeR family of proteins. Sequence analysis of *ideR* (MAP2827) of diverse MAP strains revealed that, *ideR* ORF and IdeR regulated promoters exhibit polymorphisms that could lead to functional variation between cattle and sheep strains.

It is believed that *M. avium* encompasses a diverse group of subspecies (18), that are host specific and that this specialization occurred after diversification. Molecular epidemiological studies, comparative genomic hybridizations and host-pathogen interaction studies suggest that MAP is genotypically and phenotypically diverse (9, 10, 12). It is also now well-established that sheep MAP strains are extremely slow growing organisms in routine laboratory cultures relative to cattle MAP strains. Sequence analysis of the ideR ORF and the promoters of IdeR regulated genes demonstrated polymorphisms between cattle and sheep MAP strains. Functional studies with two IdeR regulated genes *mbtB* (iron acquisition gene) and *bfrA* (iron storage gene) revealed variations in *bfrA* regulation under adequate iron conditions. This variation may partially explain their in vitro phenotype in that the sheep strain may have a defective iron storage pathway leading to iron toxicity when excess iron is provided in culture media. More directed studies on iron storage mechanisms in sheep strains would be needed to fully address this contention.

It is counterintuitive for sheep strains to repress transcription of an iron storage gene under high iron conditions that are also expected to occur in an early phagosome environment (19, 20). Whether differential iron storage/regulation between sheep and cattle MAP exist or not would be better addressed when the genome sequence of a sheep strain of MAP is available for analysis.

Transcriptional start point (TSP) assays have identified two promoter sites (P_{low} and P_{high}) in the MTB *bfrA* intergenic region (6). It was proposed that in presence of iron, *bfrA* transcription was either downregulated (via P_{low}) or upregulated (via P_{high}). Our data

suggested that cIdeR in the presence of cattle MAP *bfrA* promoter upregulated transcription in presence of iron, likely behaving as P_{high}. On the other hand, IdeR from sheep or cow strains of MAP (sIdeR or cIdeR) or MTB IdeR downregulate *bfrA* transcription in presence of sheep MAP promoter under high iron conditions, suggesting a P_{low} activity. This may be due to polymorphisms identified in *bfrA* promoter of sheep MAP strains. Thus it is possible that there are alternate promoters for cattle and sheep *bfrA* that needs to be tested in future studies.

The current study was aimed at determining if *ideR* of MAP encodes for a functional protein. Our results show that MAP2827 controls transcription of genes involved in iron regulation.

*The findings presented in this chapter (Chapter 3) were published in Microbiology 155 (2009), 3683-3690.

Table 1: Iron concentration dependent expression of mbtB and bfrA as measured by Q-RT PCR in cattle and sheep strains of MAP.

		Fold Change (IE/ID)[a]
Derepressed by IdeR and iron		
	c*bfrA*	2.63
Repressed by IdeR and iron		
	s*bfrA*	0.13
	c*mbtB*	0.40
	s*mbtB*	0.17

[a] Fold change for each target gene is calculated as described in materials and methods and presented as ratio of expression under iron excess (IE) to the gene expression under iron depletion (ID). A fold change of 1 is considered no change in gene expression in response to iron where as a fold change greater than or less than 1 is considered as derepressed or repressed respectively in response to iron.

Table 2: Putative IdeR regulated genes expressed in primary human macrophages or in broth cultures

		Fold Change [a]	
ORF ID	**Predicted function**	***In-vivo***	***In-vitro***
MAP0024c	hypothetical protein		-5.14
MAP0025	acyl carrier protein		-8.20
MAP0913c	*fadE13* (fatty acid metabolism)		-7.64
MAP1559c	Transcriptional repressor	1.61	3.38
MAP1560	hypothetical protein		-7.42
MAP1595	*bfrA*		9.11
MAP1762c	Iron permease FTR1 family protein		-7.42
MAP2178	*mbtA*		-7.94
MAP2206	Putative permease protein	-4.73	-3.18
MAP3778	ATPase, AAA family protein	2.18	-2.44
MAP2173c	*mbtE*	2.05	

[a] Fold change expressed as normalized $\log_2$ values.

A blank value represents <1.5 fold change for that target

Figure 3: MAP IdeR in presence of nickel binds the predicted promoters (*mbtB* and *bfrA*) carrying iron box

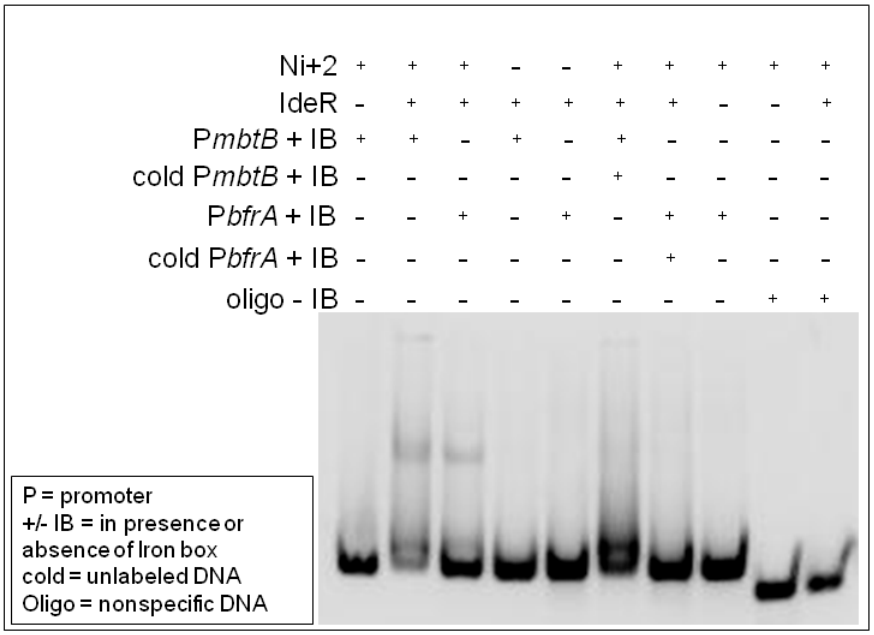

Figure 3: Electrophoretic mobility shift assay to demonstrate specificity of MAP2827 protein with the predicted promoters. Recombinant MAP2827 was reacted with *mbtB* and *bfrA* promoters for this assay. MAP IdeR reacts with the promoters only in presence of nickel (Lane 2 and 3).

Figure 4: MAP IdeR protects the iron box sequence from DNase I digestion

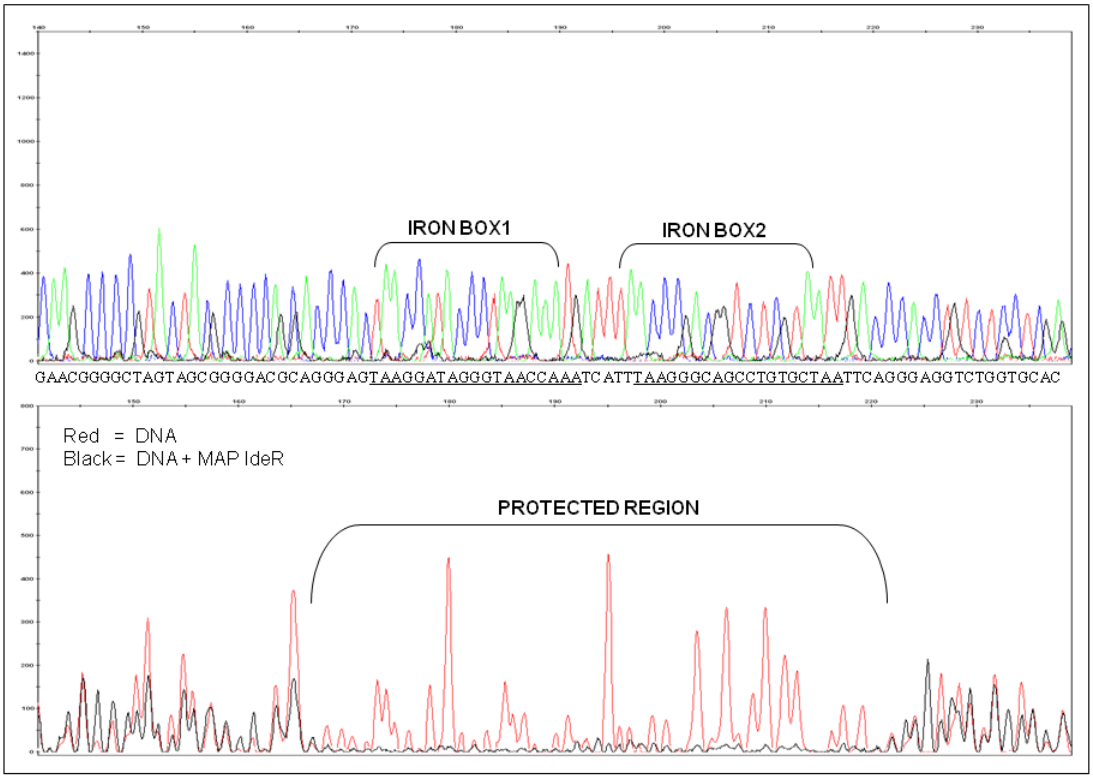

Figure 4: DNase footprint assay showing the binding of MAP2827 protein to the iron box sequence located on MAP *mbtB* promoter. Red peaks in the electropherogram represent DNA alone and black peaks represent DNA pre-incubated with MAP2827 (bottom panel). Both reactions were partially digested with DNase I and analyzed by capillary electrophoresis in a genetic analyzer (Applied Biosystems 3130xl). Shown in the top panel is the electropherogram of the promoter sequence with the terminal bases identified (iron box sequence is underlined). The protected region which overlaps the iron

box sequence is indicated. Note the difference in the heights of black peaks (lost completely) relative to black peaks indicating protection from DNase digestion.

Figure 5: Genotypes of Mycobacterial strains used in the reporter assays

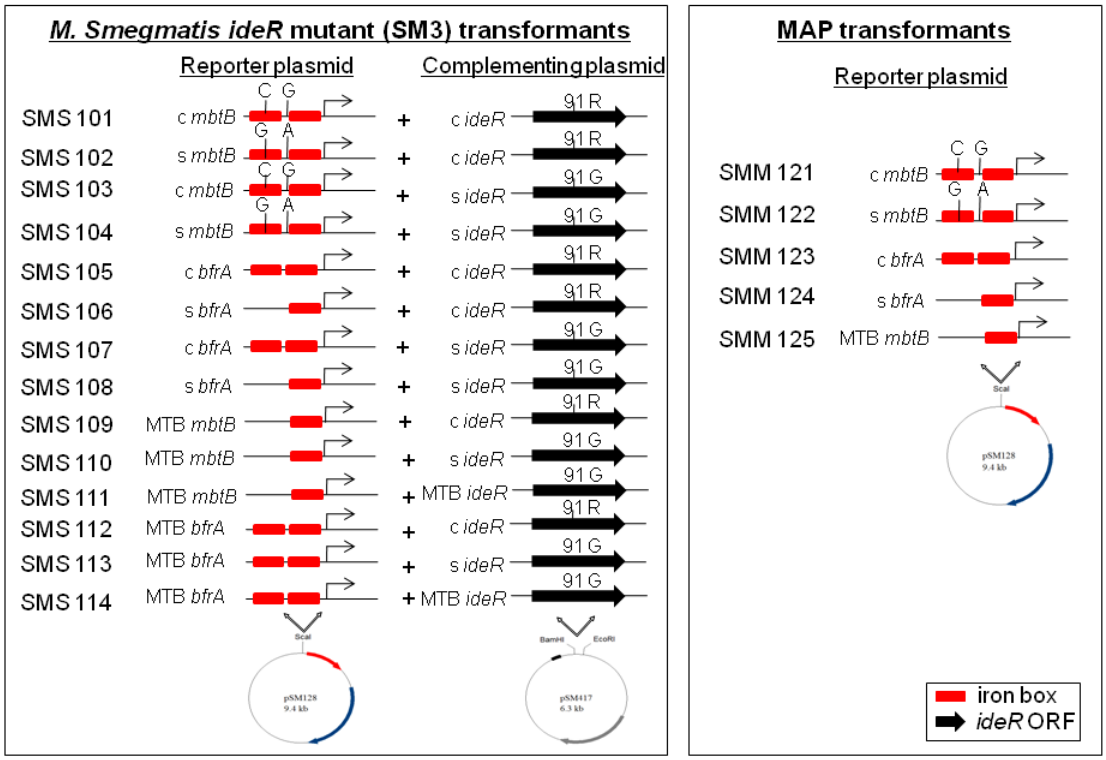

Figure 5: Depicted are the genotypes of *M. smegmatis* or MAP transformants used in reporter assays. Also highlighted are the polymorphisms present in the promoter regions of MAP *mbtB* and *bfrA* promoters.

Figure 6: Reporter activity of MTB and MAP *mbtB* promoters in an *ideR* deletion mutant of *M. smegmatis*

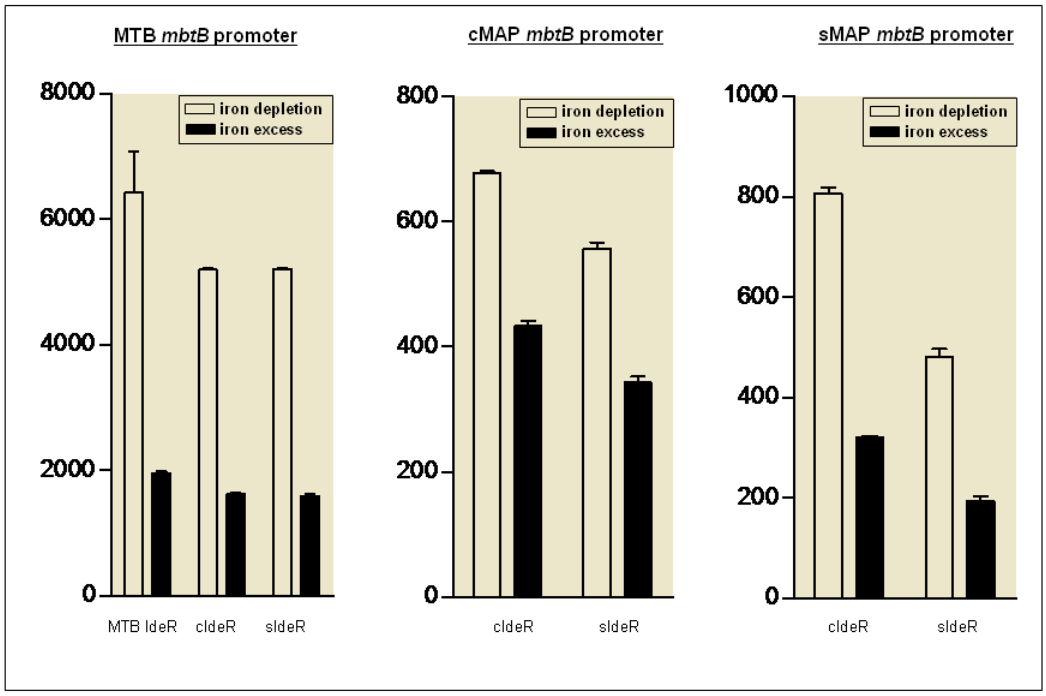

Figure 6: Reporter activity of *mbtB* promoter fusions of MTB *mbtB*, cattle MAP strain *mbtB*, or sheep MAP strains to *lacZ* driven by cIdeR or sIdeR or MTB IdeR is shown. Under iron rich conditions cIdeR, sIdeR and MTB IdeR repressed transcription of *mbtB*. Open bars transcription under iron deplete medium and closed bars indicate iron rich medium. Averages of three independent experiments each analyzed in triplicate are shown.

Figure 7: Reporter activity of MTB and MAP *bfrA* promoters in an *ideR* deletion mutant of *M. smegmatis*

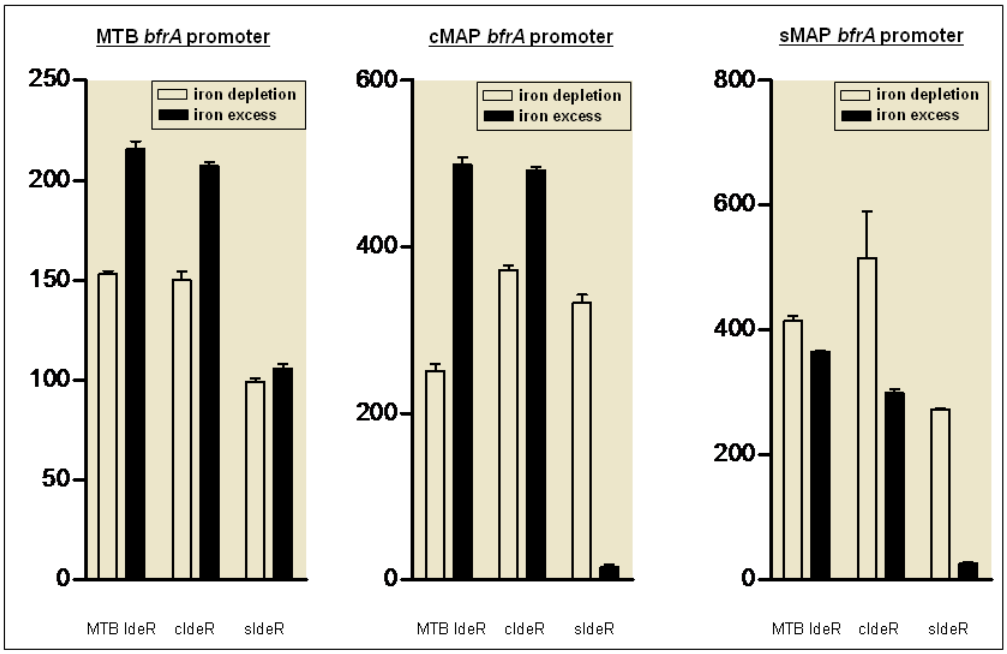

Figure 7: Reporter activity of *bfrA* promoter fusions of, MTB *bfrA*, cattle MAP *bfrA* or sheep MAP to *lacZ* driven by cIdeR or sIdeR or MTB IdeR. Under iron rich conditions cIdeR, sIdeR and MTB IdeR showed repressed transcription with sheep *bfrA* promoter. Shown in open bars is transcription under iron deplete conditions and closed bars indicate iron rich medium. Averages of three independent experiments, each performed in triplicate is shown.

Figure 8: Reporter activity of MAP *mbtB* and *bfrA* promoters in two diverse MAP strains

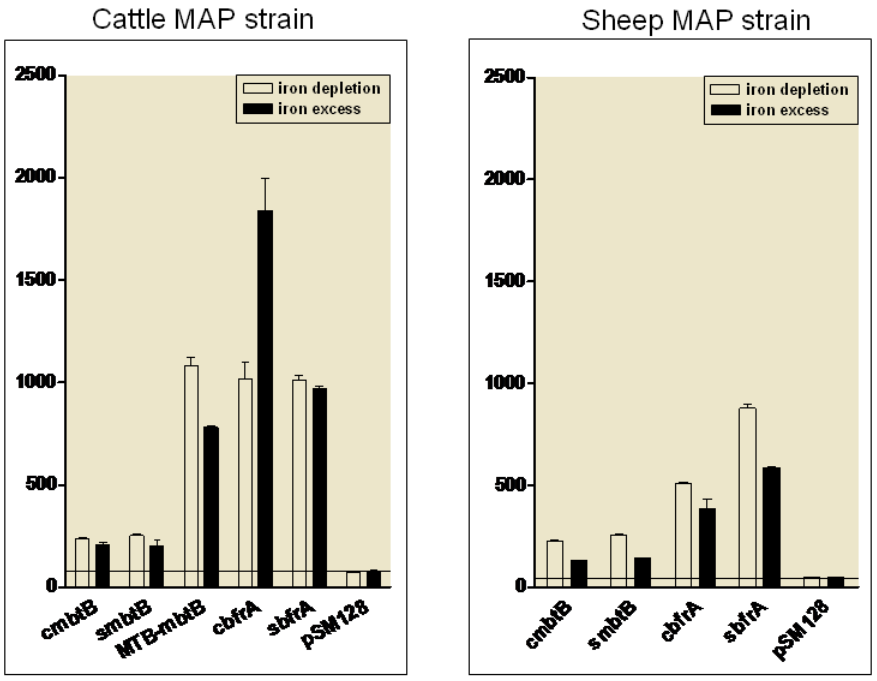

Figure 8: Reporter activity of *mbtB* and *bfrA* promoter fusions to *lacZ* in a cattle MAP strain. Line indicates the background reporter activity of empty vector (pSM128). Both *mbtB* and *bfrA* are active in MAP. The activity of MAP *mbtB* promoters is lower in comparison to that of MTB *mbtB*. *bfrA* promoter from sheep strain of MAP (*sbfrA*) showed consistently lower activity under high iron conditions as was shown in SM3 background. Open bars indicate transcription iron depleted conditions and closed bars indicate iron rich medium. Averages of three independent experiments, each performed in triplicate is shown.

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CHAPTER 4: IRON-SPARING RESPONSE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IS STRAIN DEPENDENT

Two genotypically and microbiologically distinct strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) exist – the type I and type II strains that primarily infect sheep and cattle, respectively. Concentration of iron in the cultivation medium has been suggested as one contributing factor for the observed microbiologic differences. We recently demonstrated that type I strains have defective iron storage systems, leading us to propose that these strains might experience iron toxicity when excess iron is provided in the medium. To test this hypothesis, we carried out transcriptional and proteomic profiling of these MAP strains under iron-replete or –deplete conditions. We first complemented *M.smegmatis*Δ*ideR* with IdeR of type II MAP or that derived from type I MAP and compared their transcription profiles using *M. smegmatis* mc^2155 microarrays. In the presence of iron, type I IdeR repressed expression of *bfrA* and MAP2073c, a ferritin domain containing protein suggesting that transcriptional control of iron storage may be defective in type I strain. We next performed transcriptional and proteomic profiling of the two strain types of MAP under iron-deplete and –replete conditions. Under iron-replete conditions, type II strain upregulated iron storage (BfrA), virulence associated (Esx-5 and antigen85 complex), and ribosomal proteins. In striking contrast, type I strain downregulated these proteins under iron-replete conditions.. iTRAQ (isobaric tag for relative and absolute quantitation) based protein quantitation resulted in

the identification of four unannotated proteins. Two of these were upregulated by a type II MAP strain in response to iron supplementation. The iron-sparing response to iron limitation was unique to the type II strain as evidenced by repression of non-essential iron utilization enzymes (aconitase and succinate dehydrogenase) and upregulation of proteins of essential function (iron transport, [Fe-S] cluster biogenesis and cell division). Taken together, our study revealed that type II and type I strains of MAP utilize divergent metabolic pathways to accommodate in vitro iron stress. The knowledge of the metabolic pathways these divergent responses play a role in are important to 1) advance our ability to culture the two different strains of MAP efficiently, 2) aid in diagnosis and control of Johne's disease, and 3) advance our understanding of MAP virulence.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD) of ruminants, requires siderophore (mycobactin) supplementation for optimal growth in laboratory media. This unique iron requirement makes MAP fastidious, often requiring eight to sixteen weeks to see colonies in culture – a major hurdle in the diagnosis and therefore in implementation of optimal control measures. Unlike other mycobacteria, which mobilize iron via mycobactins, MAP is unable to produce detectable mycobactin in vitro or in vivo (19, 20, 45). Although the reasons for the in vitro mycobactin dependency of MAP are currently unknown, we have recently shown that the mycobactin (*mbt*) operon promoter is active and that the mycobactin

genes are transcribed by MAP inside macrophages (18) and in tissues of naturally infected animals (submitted for publication).

Pathogenic mycobacteria encounter a wide variety of stressors inside the host cells and their ability to overcome iron deprivation and iron toxicity represents a major virulence determinant (49). Transcript and protein profiling of MTB and other pathogens in response to in vitro iron stress is well documented (14, 34, 35, 37). While MAP transcriptome or proteome profiles in response to heat shock, pH, oxidative stress, hypoxia, and nutrient starvation have been demonstrated (11, 12, 53), stress responses to iron supplementation or starvation are lacking.

Iron dependent regulator (IdeR) has been very well studied as a global regulator involved in maintaining iron homeostasis in *Mycobacterium tuberculosis* (MTB) (38). Recently we have demonstrated that IdeR of MAP in the presence of iron recognizes a consensus sequence on the promoter called “iron box” and regulates expression of genes involved in iron acquisition (*mbt*) and storage (*bfrA*). More interestingly, we demonstrated that polymorphisms in the promoter of iron storage gene (*bfrA*) in type I (sheep) MAP strains relative to type II (cattle) MAP strains results in a differential gene regulation (18). IdeR dependent repression of *bfrA* in the presence of iron suggests variations in iron storage mechanisms and/or iron requirements in cattle and sheep MAP strains.

Comparative genomic hybridizations, short sequence repeat analysis and single nucleotide polymorphisms of MAP isolates obtained from diverse host species have established and indexed genomic differences between types I and II strains of MAP (22,

23, 30-33). Phylogenetic analysis of sequences have identified type I and type II strains as separate pathogenic clones that share a common ancestor (2, 47, 48, 52). Furthermore, cellular infection studies show distinctive phenotypes between the two MAP strain types (17, 28). We also recently demonstrated that type I strains have defective iron storage systems, leading us to propose that these strains might experience iron toxicity when excess iron is provided in the medium (18). Taken together, the literature suggests that MAP strains vary in their iron dependent gene regulation. To test this further, we profiled their transcriptomes and proteomes in response to iron and demonstrated that iron induced metabolic pathways are significantly diverse.

MATERIALS AND METHODS

Bacterial strains, DNA manipulations and media: *Mycobacterium avium* subsp. *paratuberculosis* strains MAP1018 (type II) and MAP7565 (type I) were grown in Middlebrook 7H9 supplemented with OADC enrichment medium and mycobactin J (2mg/mL; Allied Monitor, Fayette, MO). MAP7565 and MAP1018 have been genotyped by SSR as well as comparative genomics using oligoarrays. They represent the typical genomotypes of sheep and cattle strains, respectively (33) and show distinct phenotypes in both human and bovine macrophages (17, 28).

M. smegmatis (mc^2155) and *E. coli* TOP10F (Invitrogen Corporation, Carlsbad, CA) competent cells were grown in Luria Bertani (LB) medium and antibiotics (kanamycin (20 µg/ml) or hygromycin (100 µg/ml)) were added when necessary. The

open reading frames of *ideR* (MAP2827) derived from type I or type II MAP strains were cloned into pSM417 and *M. smegmatis*Δ*ideR* (SM3) was complemented as previously reported (18). Briefly, MAP2827 from MAP1018 (c*ideR*) or MAP 7565 (s*ideR*) was amplified via PCR using primers that carried restriction sites for *BamHI* and *HindIII*. Amplified products were double digested with *BamHI* and *HindIII* and ligated into a pre digested (*BamHI* and *HindIII*) expression plasmid pSM417. Accuracy of the ligation and orientation of MAP2827 in pSM417 was verified by sequencing. SM3 was transformed with pSM417 carrying MAP2827 from type I or type II MAP strains.

All bacterial strains were grown to logarithmic growth phase and iron limitation was carried out by addition of 2,2'-dipyridyl (Sigma Aldrich, St. Louis, MO) at a concentration of 200 µM. The detailed experimental design is provided as Figure S2.

Nucleic acid and protein extraction: Log phase MAP or *M. smegmatis* cultures were pelleted, washed and re-suspended in fresh culture medium with or without 200 µM of 2,2'-dipyridyl. The cultures were incubated at 37°C with shaking for 3 h immediately prior to RNA and protein extraction.

For RNA, cells were homogenized in Mini bead-beater for 4 min by adding 0.3ml of 0.1mm sterile RNase-free zirconium beads followed by extraction using Trizol (Invitrogen, Carlsbad, CA). All samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to eliminate genomic DNA contamination. The purity and yield of total RNA samples was confirmed using Agilent 2100E Bioanalyzer (Agilent Technologies,

Inc., Santa Clara, CA). RNA was stored at -80 until used in microarrays and real time RT-PCR assays.

For protein, cells were re-suspended in minimal quantity (250 μL) of iTRAQ dissolution buffer (0.5 M TEAB pH 8.5) and 0.1% SDS. The solution was transferred to a 2 ml screw cap tube containing 0.1mm zirconium beads (Biospec) and disrupted in minibead beater (Biospec) for 4X1 minute pulses with samples kept on ice every minute. The lysate was then centrifuged at 12,000xg for 10 minutes at 4°C. Supernatant was transferred to a fresh tube without disturbing the pellet and used in iTRAQ labeling for detection of proteome (Figure S2).

Microarray experiments: Gene expression profiling of type II (1018) and type I (7565) MAP strains was performed using MAP K-10 microarrays obtained from Dr. Michael Paustian, NADC, IA. Expression profiling of *M. smegmatis*Δ*ideR* complemented with c or s*ideR* was carried out using *M. smegmatis* mc^2155 arrays provided via Pathogen Functional Genomics Resource Center (PFGRC) at J. Craig Venter Institute (JCVI). Array hybridizations and analyses were performed as described previously and according to the protocols established at PFGRC with minor modifications (39).

Briefly, synthesis of fluorescently labeled cDNA (Cyanine-3 or Cyanine-5) from total RNA and hybridizations of labeled cDNA to MAP K-10 or mc^2155 oligoarray was performed. Each slide was competitively hybridized with cDNA obtained from iron-replete (labeled with cy3 or cy5) and iron-limiting growth medium (counter labeled with cy5 or cy3) to reveal relative expressional differences. Microarray hybridizations were

performed from cDNA isolated from two independent experiments. Hybridized slides were scanned using HP Scan array 5000 (PerkinElmer Inc., Waltham, MA). The images were processed and numerical data was extracted using the microarray image analysis software, BlueFuse (BlueGnome Ltd, Cambridge) and TM4 microarray suite available through JCVI. Genes differentially regulated at a fold change of 1.5 or greater were identified at a false discovery rate of 1% by Statistical Analysis of Microarrays (SAM) program (39).

Realtime RT-PCR: RNA isolated from MAP strains grown under iron-replete or iron-limiting growth medium was used in real time RT-PCR assays. Genes were selected based on their diverse roles and microarray expression pattern. Selected genes included siderophore transport (MAP2413c, MAP2414c), *esx-3* secretion system (MAP3783, MAP3784), aconitase (MAP1201c), fatty acid metabolism (MAP0150c) and virulence (MAP0216, MAP3531c, MAP1122 and MAP0475). RNA was treated with DNaseI (Ambion, Austin, TX) and Q-RT PCR was performed using QuantiFast SYBR Green mix (Qiagen, Valencia, CA) and gene specific primers (Table S2) in a Lightcycler 480 (Roche, Indianapolis, IN).

iTRAQ experiments: Protein extracted from the two MAP strains grown in iron-replete or iron-limiting medium was used in iTRAQ analysis (Figure S3). iTRAQ labeling and protein identification was carried out as described previously with minor modifications (42).

Briefly, cell lysate was quantified using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) prior to trypsin digestion. Peptides were labeled with iTRAQ reagents (114 and 115 for MAP 1018 grown in iron-replete and iron-limiting medium respectively; 116 and 117 for MAP 7565 grown in iron-replete and iron-limiting medium respectively) at lysine and arginine amino terminal groups. The labeled peptides were pooled, dried and re-suspended in 0.2% formic acid. The re-suspended peptides were passed through Oasis® MCX 3CC (60 mg) extraction cartridges per manufacturer recommendations (Waters Corporation, Milford, MA) for desalting prior to strong cation exchange (SCX) fractionation.

Eluted peptides were dried and dissolved in SCX buffer A (20% v/v ACN and 5 mM KH_2PO_4 pH 3.2, with phosphoric acid) and fractionated using a polysulfoethyl A column (150 mm length × 1.0 mm ID, 5 μm particles, 300 Å pore size) (PolyLC Inc., Columbia, MD) on a magic 2002 HPLC system (Michrom BioResources, Inc., Auburn, CA). Peptides were eluted by running a 0-20% buffer B gradient for greater than 55 min. and 20%-100% buffer B (20% v/v ACN, 5 mM KH_2PO_4 pH 3.2, 500 mM KCL) for 20 min. at a column flow rate of 50 μl / min. Several fractions were collected at frequent intervals and seven fractions that showed mAU280 > 2 were analyzed by LC-MS/MS as previously described.

Fractions were reconstituted in reversed-phase load buffer (10mM phosphate buffer) and analyzed in a 4800 MALDI TOF/TOF instrument (AB Sciex, Foster city, CA). Protein pilot Software™ 3.0.1 (AB Sciex, Foster City, CA) was used to identify and quantify the relative abundance of the labeled peptides which utilizes the paragon™

scoring algorithm. Relative abundance of proteins (iron-replete v/s iron-limitation) for each MAP strain was determined by comparing the reporter ion ratios (114/115 for C and 116/117 for S MAP). iTRAQ experiments were repeated on two independent experiments for each treatment of each strain. We searched against the entire non-redundant (nr) protein database deposited in the NCBI along with the contaminants to identify MAP specific peptides at a false discovery rate of 1%.

RESULTS

Transcriptional profiling of MAP IdeR in *M. smegmatis:* We recently characterized MAP IdeR and computationally predicted that IdeR in the presence of iron regulates expression of 24 genes (18). We identified that 20 of the 24 predicted genes were differentially expressed in response to iron. Mycobactin synthesis, transport and fatty acid biosynthesis genes were repressed in the presence of iron by both cattle and sheep MAP strains (Table S3). However iron storage and oxidoreductase genes were upregulated in the presence of iron only in type II MAP (Figure 1).

We first confirmed if these differences are due to regulation via IdeR. *ideR* is essential in MAP and attempts to delete this gene failed (39). We complemented *M. smegmatis*Δ*ideR* (SM3) with type II or type I strain *ideR* and compared regulational differences in the presence or absence of iron. Genes that showed a $\log_2$ fold change of 1.0 in SM3 in the presence or absence of iron while having a fold change >± 1.5 (after normalized against controls) in the complemented strains were considered as being regulated by MAP IdeR. Controls used were SM3 complemented with empty plasmid,

plasmid carrying *M. smegmatis ideR* and *mc^{2}155* (wild type). Fourteen of the 20 genes were regulated by IdeRs of both MAP strains in *M. smegmatis.* Furthermore, our results suggested that type I IdeR functions by primarily repressing genes in the presence of iron whereas type II IdeR functions both by repressing mycobactin synthesis and de-repressing iron storage genes in the presence of iron (Table S4). These were further validated by realtime RT-PCR (Table S5).

We next compared the transcriptome and proteomes of type I and type II MAP strains under iron-replete and iron-limiting conditions.

Transcript profiles under iron-limiting conditions: Under iron-limiting conditions both the MAP strains showed increased transcription of genes belonging to mycobactin synthesis and esx-3, an essential secretory system of mycobactin biosynthesis (Tables S3 – S6) (43).

Type II MAP showed increased transcription of genes belonging to ABC type transporter proteins, *suf* operon involved in Fe-S cluster assembly proteins (MAP1187-MAP1192), fatty acid biosynthesis operon (MAP3188-MAP3190) and a pyruvate dehydrogenase operon (MAP2307c-MAP2309c) (Table 5 and S6) suggesting that the transcriptional machinery is used to mobilize iron to maintain intracellular homeostasis. Cattle MAP (type II strain) also upregulated expression of an enhanced intracellular survival gene (*eis*) (MAP2325), which was described as “deletion 3” in sheep strains of MAP (22).

In contrast, the sheep strain of MAP in addition to upregulation of putative iron uptake and transport genes also expressed those belonging to heat shock proteins, molecular chaperones, and a VapBC family of toxin-antitoxin operon (MAP2027c, MAP2028c) suggesting that iron deprivation might lead to a stringency response (Table 4 and S7).

Transcript profiles under iron-replete conditions: There is increased protein synthesis and turnover in response to iron in *M. tuberculosis* (MTB) (36). Similarly, the cattle strain (type II) upregulated as many as 25 rRNA genes, lipid metabolism, and several virulence-associated genes such as *fbpA* (MAP0216) of antigen85 complex, soluble secreted antigen (MAP2942c), and oxidoreductase (MAP1084c) (Tables 5 and S8). There was also an upregulation of MAP3296c, a *whiB* ortholog of *M. tuberculosis* that plays a role in antibiotic resistance and maintains intracellular redox homeostasis (44). Further, esx-5 operon which is present only in pathogenic mycobacteria and plays a role in cell-cell migration of mycobacteria was upregulated (1). A hypothetical protein (MAP0860c) upregulated in the presence of iron in the cattle strain of MAP has been described as a part of MAP-specific large sequence polymorphism (LSP4) (2).

In contrast, we did not document any upregulation (at a $\log_2$ fold change of 1.5) in the type I MAP under iron-replete conditions. The directionality of transcripts as identified by microarrays under iron-replete conditions by type I MAP strain were confirmed by real time RT-PCR (Table S5).

Proteome: The following criteria were used for protein identification in each treatment - (1) peptides identified by mass spectrometry were searched against the non-redundant (nr) protein database deposited in NCBI); and (2) MAP specific peptides reported with >95% confidence were used to quantify the relative abundance (iron-replete v/s iron-limitation) of each protein. A peptide with no hits on the MAP genome but with identities with other mycobacterial proteins was considered as unannotated MAP protein.

Five proteins with a (fold change >1.5) has BLAST hits against *Aspergillus fumigatus* proteins in the nr database search. However, these were not significant ($P>0.05$) (not shown). Of the 300 MAP proteins identified at a false discovery rate of 1%, 64 and 60 proteins had a fold change of 1.5 or greater in type II or type I MAP culture extracts, respectively. In the presence of iron, one third of the differentially regulated genes ($P<0.05$) were represented both in the respective transcriptome and the proteomes of the two strains (Figure 10).

Protein expression under iron-limiting conditions: Consistent with the transcription profile, the type II strain of MAP upregulated proteins belonging to SUF operon involved in Fe-S cluster assembly, fatty acid metabolism and a pyruvate dehydrogenase (MAP2307c). Transporter proteins, two component systems, and cell division associated proteins (MAP1906c, MAP0448 and MAP2997c) were also upregulated by the type II strain (Table 5 and S9). The sheep (type I) strain also upregulated transporter proteins, fatty acid biosynthesis, DNA replication protein

(MAP3433), and stress response proteins (MAP3831c, MAP2764) (Table 4, S10 and Figure S4).

The iron-sparing response to iron starvation occurs when non-essential iron utilization proteins such as aconitase and succinate dehydrogenases are repressed and intracellular iron is used to maintain essential cellular functions (10, 16). Interestingly, during iron limitation, the cattle strain but not sheep MAP downregulated expression of aconitase (MAP1201c) and succinate dehydrogenases (MAP3697c, MAP3698c) (Figure 11). Repression of aconitase in response to iron starvation is post-transcriptionally mediated via small RNAs (24). Consistent with this finding, our results reveal an upregulation of aconitase transcripts (both by microarray and Q-RT PCR) with a concomitant downregulation at the protein level in the type II MAP alone under iron-limiting conditions.

Protein expression under iron-replete conditions: The sheep strain upregulated as many as 13 unique peptides (>95% confidence) that were mapped to MAP2121c (Figure 12). Interestingly, none of these were differentially regulated in response to iron by a type II strain of MAP. MAP2121c was originally described as 35-kDa antigen and is an immune-dominant protein involved in MAP entry into bovine epithelial cells (3, 4). Although statistically not significant, further microarray analysis revealed a two-fold increase of MAP2121c in both cattle and sheep strains under iron-replete conditions (data not shown), suggesting a possible post transcriptional repression of MAP2121c by the cattle strain of MAP.

As expected, transcripts identified as upregulated under iron-replete conditions in type II MAP strain were also upregulated in the proteome (Table 5, S10 and Figure S5). There was increased expression of five ribosomal proteins and a ribosome releasing factor (MAP2945c) by cattle MAP under iron-replete conditions. As previously reported, BfrA was upregulated in cattle MAP (Figure 13). Antigen 85A and MAP0467c (mycobacterial heme, utilization and degrader) were also upregulated. However, MAP0467c and other stress response proteins were downregulated in the type I MAP strain (Figure 14).

Identification of unannotated MAP proteins: We identified two unique peptides (**SSHTPDSPGQQPPKPTPAGK** and **TPAPAKEPAIGFTR**) that originated from the unannotated MAP gene located between MAP0270 (*fadE36*) and MAP0271 (ABC type transporter) (Figure 15A). We also identified two peptides (**DAVELPFLHK** and **EYALRPPK**) that did not map to any of the annotated MAP proteins but to the amino acid sequence of MAV_2400. Further examination of the MAP genome revealed that the peptides map to the reversed amino acid sequence of MAP1839 (Figure 15B). These two unique proteins were not differentially regulated in response to iron. However, two more unique peptides that were translated from other unannotated MAP genes were upregulated (>1.5 fold) under iron-replete conditions in C MAP strain (Figure 16 A and B).

DISCUSSION

Johne's disease is a major animal health problem of ruminant species worldwide and imposes significant economic losses to the industry. Our ability to culture the causative agent--*Mycobacterium avium* subsp. *paratuberculosis* (MAP)--and therefore its rapid diagnosis and our understanding of its virulence is limited. MAP is difficult to culture because of its unusually strict iron requirements. For optimal growth in laboratory media, MAP requires a siderophore (mycobactin) supplementation that makes MAP fastidious (26)., often requiring eight to sixteen weeks to produce colonies in culture – a major hurdle in the diagnosis and therefore implementation of optimal control measures. Understanding iron regulatory networks in the pathogen in vitro is therefore of great importance.

A tale of two strain types of MAP – A case to study iron regulation: Several microbiological and genotyping studies and clinical observations suggest that Johne's in certain hosts such as sheep, goats, deer, and bison is caused by a distinct set of strains that show a relatively high degree of host preference (29, 33). At least two microbiologically distinct types of MAP have been recognized. A less readily cultivable type is the common, but not invariable, cause of paratuberculosis in sheep (type I) (13, 26, 51), while another readily cultivable type is the most common cause of the disease in cattle (type II). Cell infection studies have also revealed distinctive host response phenotypes between type I and type II strains - the former elicit primarily a pro-inflammatory response while latter strains suppress inflammation while upregulating anti-apoptotic pathways (17, 28). In addition, since MAP genome sequence was published in 2005, very

little research has focused on iron physiology and its contribution to metabolic networks of this fastidious organism.

Based on these classical microbiologic, genotypic, and clinical observations, we addressed the hypothesis that the iron dependent gene regulation is different between type I and type II MAP strains using a systems approach.

Iron-sparing response to iron-limitation is unique to type II MAP strain: Iron is a critical component of several metabolic enzymes (50). Most bacteria respond to iron starvation with a unique regulatory mechanism called the iron-sparing response (16). Iron-sparing is a physiological phenomenon used by cells to increase the intracellular iron pool by post-transcriptionally repressing the synthesis of non-essential iron using proteins and sparing iron for essential cellular functions (25). Therefore, the paradigm is to transcriptionally upregulate all iron uptake systems while repressing non-essential enzymes via post-transcriptional regulatory mechanisms to survive iron-limiting conditions. Both MAP strains upregulated genes involved in siderophore biosynthesis (*mbt*), ability to acquire iron from synthesized siderophores (*esx-3*), and to transport iron bound siderophores (*irtAB*) into the bacterium. Furthermore, both MAP strains upregulated transcripts of aconitase and succinate dehydrogenases. However, only the cattle strain but not sheep strain showed downregulation of protein expression of aconitase and succinate dehydrogenases under iron-limitation. It is likely that targets for post-transcriptional repression of these non-essential iron using proteins are mediated via

small RNAs (10). Studies to test this hypothesis in the two MAP strain types are underway.

Differential metabolic responses of type I and type II MAP strains to iron-limitation: Under iron-limiting conditions most other bacteria including *M. tuberculosis* (MTB) upregulate SUF operon (39, 40). SUF synthesizes [Fe-S] clusters and transports them to iron-sulfur containing proteins involved in diverse cellular functions such as redox balance and gene regulation (8). This is critical because unlike *E. coli*, MTB and MAP genomes encode for only one such system to synthesize all the [Fe-S] needed by the cell and free iron and sulfide atoms are toxic to cells (15). Our data revealed that cattle strain, but not type I strain upregulated SUF operon at the transcript and protein level under iron-limiting conditions.

Type II or cattle strain upregulated pyruvate dehydrogenase operon involved in catabolism of propionate a key component of lipid biosynthesis under limiting iron conditions (41). In contrast, sheep strain upregulated isoprenoid synthesis genes involved in cell wall biogenesis (7). The sheep isolate also upregulated oxidoreductase and stress responses in its transcriptome and proteome during iron-limitation. CarD and toxin-antitoxin systems primarily function during unfavorable conditions such as starvation or oxidative stress by arresting cell growth (27, 46). Sheep strain upregulated transcripts of toxin-antitoxin system involved in arresting cell growth, suggesting a trend toward stringency response. Taken together, our data suggests that cattle strain is able to

efficiently modulate its metabolism during iron-limitation - probably a survival advantage.

MAP2325, a hypothetical protein deleted in the sheep strain was found to be upregulated under iron-limiting conditions by the type II strain. This is interesting because an ortholog of MAP2325 in MTB called *enhanced intracellular survival* (*eis*) interacts with host T cells. Stimulation of recombinant Eis from MTB results in increased production of IL-10 and decreased production of TNF-α thus contributing to mycobacterial survival inside macrophages (21). We have also demonstrated a similar result in bovine or human macrophages stimulated with diverse MAP strains. Cattle strains produced relatively more IL-10 and less TNF-α and persisted for longer periods of time inside macrophages (17, 28).

There is increased protein synthesis and turnover in response to iron in MTB (36). Similarly, we evidenced an increased expression of ribosomal proteins in the transcriptome and proteome in type II MAP under iron-replete conditions. In striking contrast, iron-limitation induced a similar theme in sheep strain. Heme degradation is a significant physiological phenomenon wherein pathogens recycle iron and gain a survival advantage inside the host (9). Recently the crystal structure of Rv3592 of MTB was solved and demonstrated its ability as heme degrader (6). We observed an upregulation of MAP0467c protein (ortholog of Rv3592) under iron-replete conditions in type II MAP while it was downregulated in the sheep strain. Similar to our previous reports, iron storage protein, BfrA was upregulated only by type II MAP under iron-repletion (18). Although the reasons for differential iron storage mechanisms in sheep compared to cattle

strains of MAP are currently unknown, differential role of ferritins in bacterial pathogens is not uncommon (5).

In summary, our data revealed differences in metabolic pathways used by cattle (type I) and sheep (type II) strains of MAP to adapt to iron starvation (Figure 17). We have identified and characterized key iron dependent pathways of MAP. Since iron metabolism is critical for the in vivo and in vitro survival of the bacterium, our current studies are expected to improve our ability to provide better in vitro culture methods for MAP and provide an understanding of iron regulation as a key virulence determinant of MAP.

Table 3: Transcript and protein expression in cattle MAP under iron-limiting (LI) conditions

	MAP ORF ID	Predicted function	[a] Fold change	
			Protein	Transcript
Metabolism				
	MAP1587c	alpha amylase	2.03	2.87
	MAP1554c	FadE33_2 (acyl-coA synthase)	1.79	1.88
	MAP2307c	pdhC	1.68	2.52
	MAP3189	FadE23	2.41	3.51
	MAP3694c	FadE5	1.87	3.15
Cellular processes				
	MAP3701c	heat shock protein	2.18	2.48
	MAP1188	FeS assembly protein SufD	2.23	2.73
	MAP1189	FeS assembly ATPase SufC	1.78	2.03
	MAP4059	heat shock protein HtpX	1.48	1.66
Poorly characterized pathways				
	MAP1012c	patatin-like phospholipase	1.67	1.56
	MAP1944c	iron sulphur cluster biosynthesis	1.56	1.66
	MAP2482	Glyoxalase resistance	1.84	2.19
	MAP3838c	RES domain containing protein	1.50	2.40

[a] MAP oligoarray was used to measure gene expression whereas iTRAQ was used to quantitate protein expression in the cultures of cattle MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of LI/HI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of transcripts and proteins in LI compared to HI. Genes are annotated based on the motif searches in KEGG database.

Table 4: Transcript and protein expression in sheep MAP under iron-limiting (LI) conditions

	MAP ORF ID	Predicted function	[a] Fold change	
			Protein	Transcript
Metabolism				
	MAP3564	methyltransferase	1.54	1.58
	MAP1942c	CbhK, ribokinase	1.74	2.05
	MAP2286c	thioredoxin domain protein	1.82	2.04
	MAP1997	acyl carrier protein	1.90	1.68
Cellular processes				
	MAP4340	TrxC, thioredoxin	1.50	2.29
	MAP3840	DnaK molecular chaperone	1.63	3.52
Information storage and processing				
	MAP4142	FusA, elongation factor G	1.52	2.58
	MAP4268c	transcriptional regulator	1.52	1.50
	MAP4233	DNA-directed RNA polymerase	1.56	1.83
	MAP3024c	DNA binding protein, HU	1.60	1.81
	MAP4184	30S ribosomal protein S5	1.75	1.55
	MAP3389c	response regulator	1.94	1.59
	MAP4111	NusG	1.98	1.82
	MAP4143	elongation factor Tu	2.08	2.16
Poorly characterized pathways				
	MAP2844	conserved hypothetical	1.54	2.27
	MAP3433	initiation of DNA replication	1.63	1.91
	MAP0126	transcriptional regulator	1.75	1.50
	MAP1065	pyridox oxidase	1.83	1.52

[a] MAP oligoarray was used to measure gene expression whereas iTRAQ was used to quantitate protein expression in the cultures of sheep MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of LI/HI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of transcripts and proteins in LI compared to HI. Genes are annotated based on the motif searches in KEGG database.

Table 5: Transcript and protein expression in cattle MAP under iron-replete (HI) conditions

	MAP ORF ID	Predicted function	[a] Fold change	
			Protein	Transcript
Metabolism				
	MAP0150c	FadE25_2	1.72	3.69
	MAP0789	acetyl-CoA acetyltransferase	1.73	2.95
	MAP1846c	ATP phosphoribosyltransferase	1.69	12.82
	MAP2332c	Fas (fatty acid synthase)	1.61	4.84
	MAP3404	AccA3	1.45	4.53
	MAP3698c	succinate dehydrogenase	1.89	23.81
Cellular processes				
	MAP1339	iron regulated protein	1.62	1.72
	MAP1653	thiol peroxidase	1.79	4.90
Information storage and processing				
	MAP2907c	translation initiation factor IF-2	1.57	3.70
	MAP2945c	ribosome releasing factor	1.66	4.31
	MAP4113	50S ribosomal protein L1	1.61	2.96
	MAP4125	rplJ 50S ribosomal protein L10	1.52	3.16
	MAP4142	fusA elongation factor G	2.13	8.30
	MAP4160	rpsJ 30S ribosomal protein S10	1.68	7.30
	MAP4181	rpsH 30S ribosomal protein S8	1.79	5.35
	MAP4233	rpoA	1.56	3.13
Poorly characterized pathways				
	MAP0216	FbpA antigen 85-A	1.87	4.46
	MAP1122	MIHF	1.73	3.99

[a] MAP oligoarray was used to measure gene expression whereas iTRAQ was used to quantitate protein expression in the cultures of cattle MAP strain grown in iron-replete (HI) or iron-limiting (LI) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of HI/LI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of transcripts and proteins in HI compared to LI. Genes are annotated based on the motif searches in KEGG database.

Figure 9: Differentially regulated genes by types I and II IdeRs

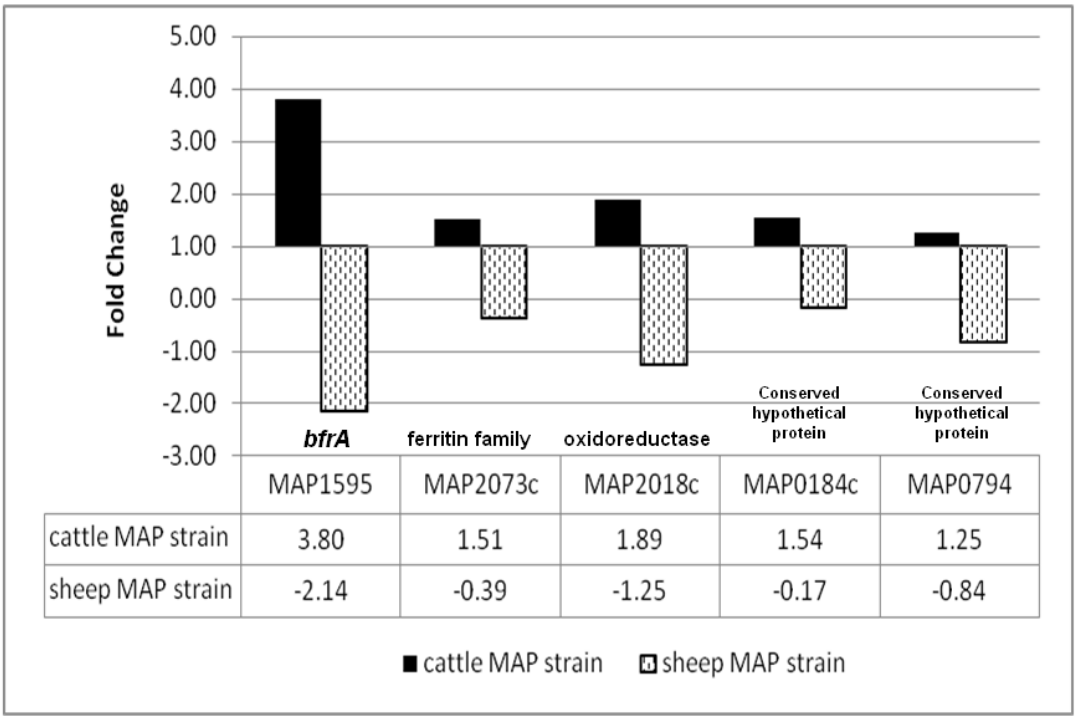


Figure 9: Transcripts upregulated in type II MAP strain while downregulated in sheep or type I MAP strain under iron-replete conditions. Fold change for each target is calculated and represented as a log2 ratio of iron-replete/iron-limitation. A negative fold change represents repression and a positive fold change indicates de-repression of that particular target gene in the presence of iron.

Figure 10: Transcriptome and proteome comparisons

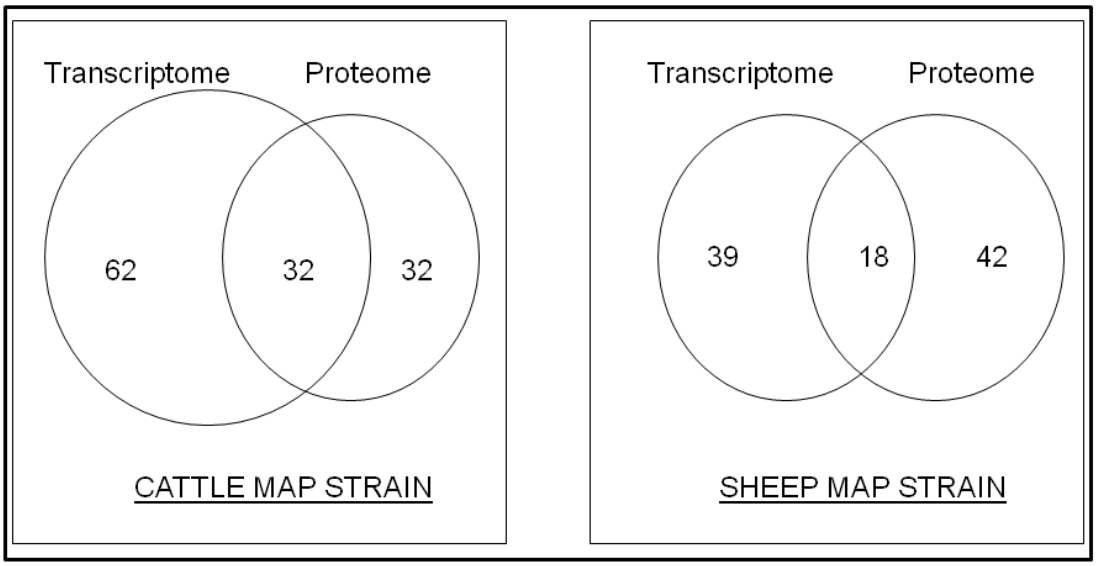


Figure 10: Venn diagram showing the comparison of transcripts and proteins that were differentially expressed at a fold change of 1.5 or greater in cattle or sheep MAP strains in response to iron. One third of the genes differentially expressed in response to iron were represented in both the transcriptome and the proteome.

Figure 11: Repression of non-essential iron using proteins under iron-limiting conditions by type II strain of MAP

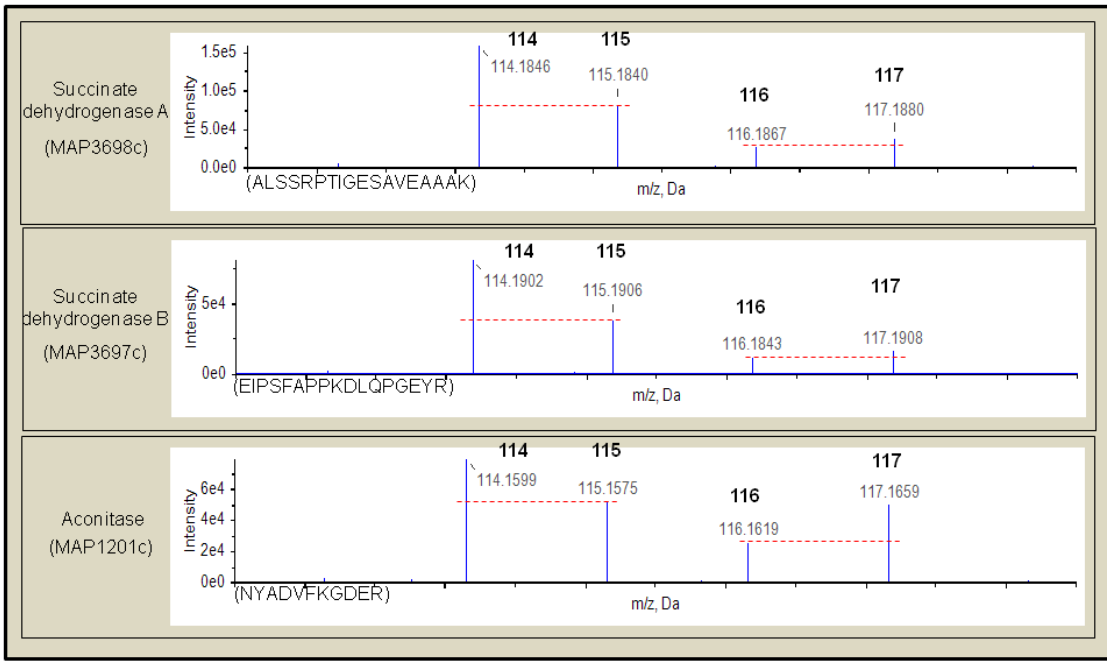


Figure 11: Reporter ion regions (114 – 117 *m/z*) of peptide tandem mass spectrum from iTRAQ labeled peptides from MAP3698c, MAP3697c and MAP1201c are shown. Quantitation of peptides and inferred proteins are made from relative peak areas of reporter ions. Peptides obtained from cattle MAP cultures grown in iron-replete and iron-limiting medium were labeled with 114 and 115 reporter ions, respectively.. Peptides obtained from sheep MAP cultures grown in iron-replete and iron-limiting medium were labeled with 116 and 117 reporter ions, respectively. The peptide sequences and shown in the parenthesis and the red dashed line illustrates the reporter ion relative peak intensities. Type II strain of MAP shows an iron sparing response by downregulating expression of iron using proteins.

Figure 12: Peptide quantitation of 35-kDa major membrane protein

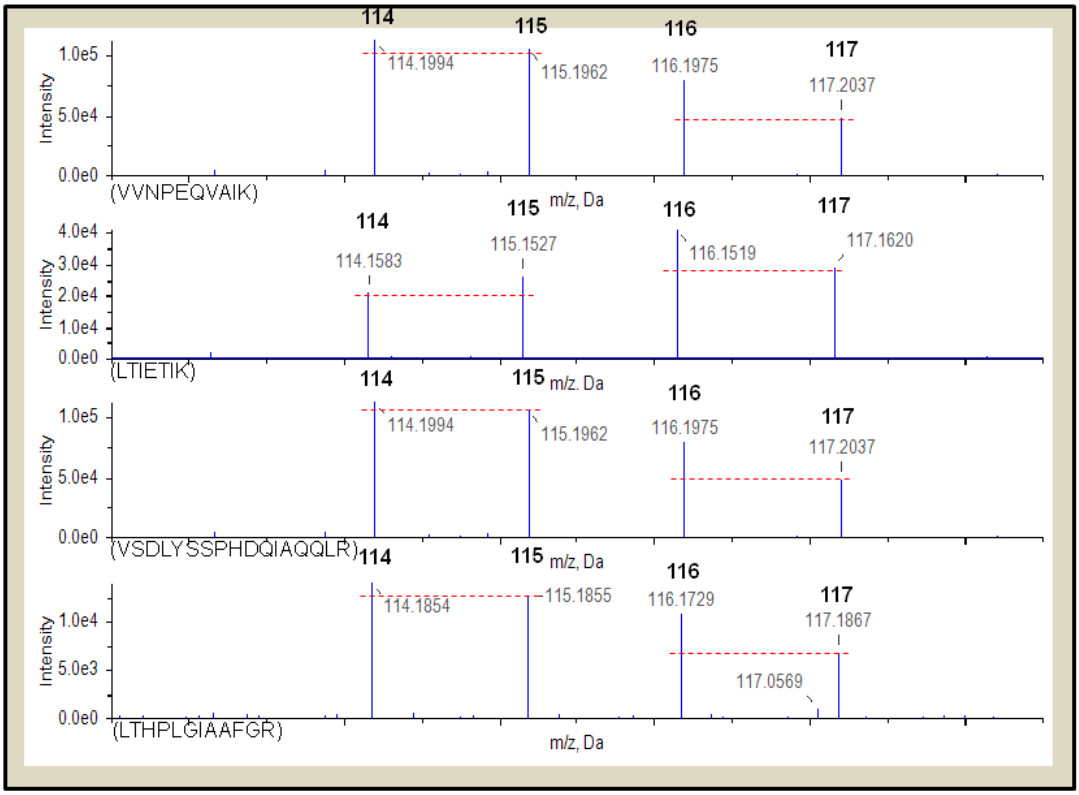


Figure 12: Reporter ion regions (114 – 117 *m/z*) of peptide tandem mass spectrum from iTRAQ labeled peptides from the 35-kDa major membrane protein (MAP2121c). Quantitation of peptides and inferred proteins are made from relative peak areas of reporter ions. Total of 13 unique peptides (>95% confidence) mapped to MAP2121c were identified but only four representative maps are shown. Peptides obtained from cattle MAP cultures grown in iron-replete and iron-limiting medium were labeled with 114 and 115 reporter ions, respectively.. Peptides obtained from sheep MAP cultures

grown in iron-replete and iron-limiting medium were labeled with 116 and 117 reporter ions, respectively. The peptide sequences and shown in the parenthesis and the red dashed line illustrates the reporter ion relative peak intensities. MAP2121c was upregulated in the sheep MAP strain under iron-replete conditions.

Figure 13: Peptide quantitation of bacterioferritin (BfrA)

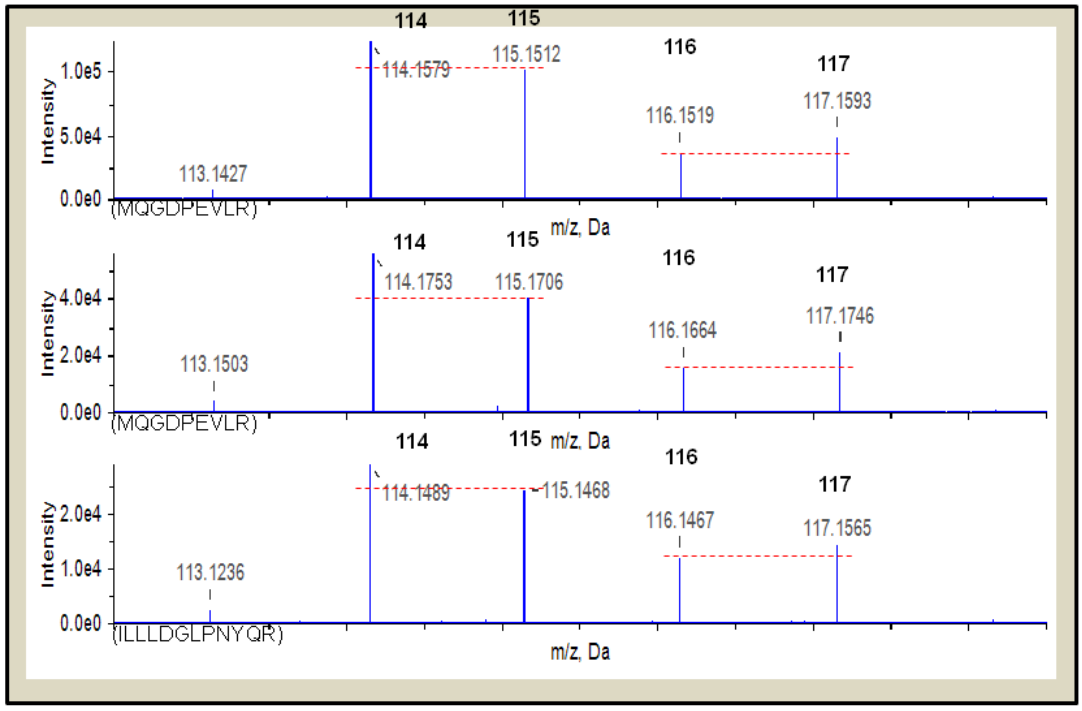
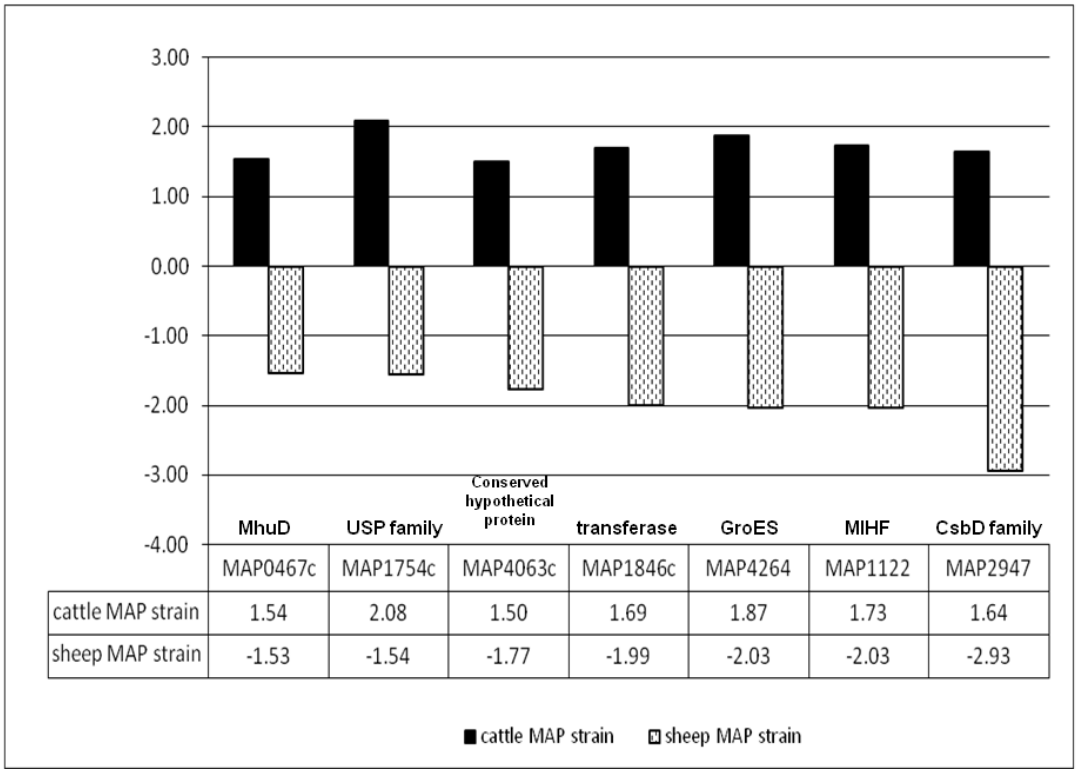


Figure 13: Reporter ion regions (114 – 117 *m/z*) of peptide tandem mass spectrum from iTRAQ labeled peptides from bacterioferritin protein (MAP1575). Quantitation of peptides and inferred proteins are made from relative peak areas of reporter ions. Total of 11 unique peptides (>95% confidence) mapped to MAP1575were identified but only three representative maps are shown. Peptides obtained from cattle MAP cultures grown in iron-replete and iron-limiting medium were labeled with 114 and 115 reporter ions, respectively. Peptides obtained from sheep MAP cultures grown in iron-replete and iron-limiting medium were labeled with 116 and 117 reporter ions, respectively. The peptide sequences and shown in the parenthesis and the red dashed line illustrates the reporter ion

relative peak intensities. MAP1575 was upregulated in the C MAP strain under iron-replete conditions.

Figure 14: Proteins expressed by type II MAP under iron-replete conditions



	MAP0467c	MAP1754c	MAP4063c	MAP1846c	MAP4264	MAP1122	MAP2947
cattle MAP strain	1.54	2.08	1.50	1.69	1.87	1.73	1.64
sheep MAP strain	-1.53	-1.54	-1.77	-1.99	-2.03	-2.03	-2.93

Figure 14: Proteins upregulated in type II MAP strain whereas downregulated in type I strain in the presence of iron. Fold change for each target is calculated and represented as a ratio of iron-replete/iron-limitation. A negative fold change represents repression and a positive fold change indicates de-repression of that particular target gene in the presence of iron. MhuD = mycobacterial heme utilization, degrader; USP = universal stress protein; CHP = conserved hypothetical protein; MIHF = mycobacterial integration host factor; CsbD = general stress response protein

Figure 15: Identification of unannotated MAP proteins

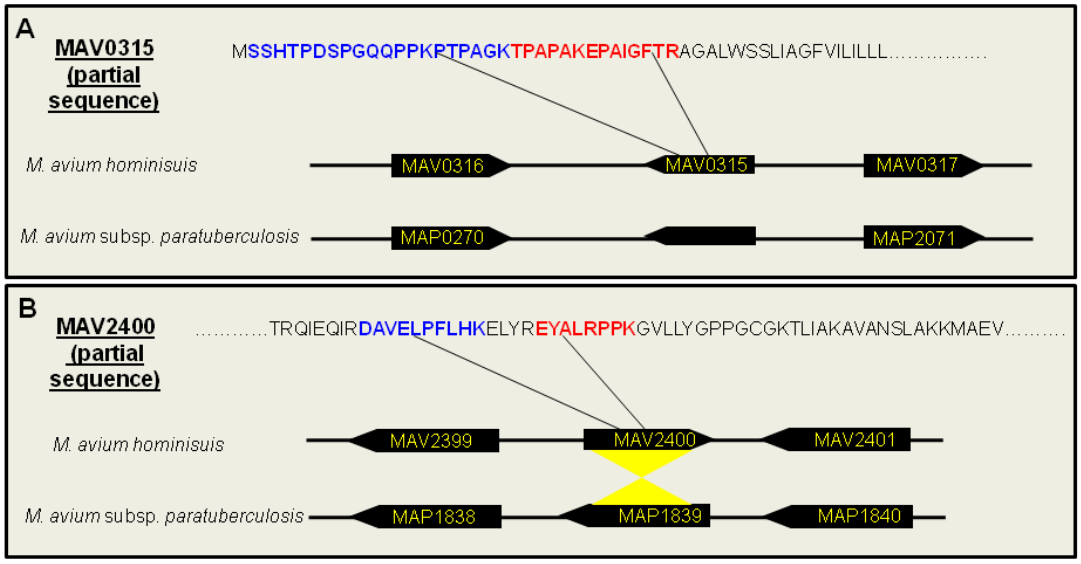


Figure 15: Shown is the linear genomic map of *M. avium* subsp. *paratuberculosis* K-10 indicating the location of peptides (indicated in color) identified by iTRAQ analysis. Respective genomic organization of *M. avium hominisuis* is also shown for comparison. (A) Identified peptides map to MAV0315 and to the intergenic region between MAP0270 and MAP0271 suggesting a missing open reading frame (ORF) in MAP K-10 genome. (B) Identified peptides map to MAV2400 but not MAP1839. However, these peptides can be identified if MAP1839 ORF is oriented in the same direction as MAV2400 suggesting that the organization of MAP1839 in MAP K-10 genome should be reversed.

Figure 16: Identification of unannotated MAP proteins upregulated exclusively by type II MAP in the presence of iron

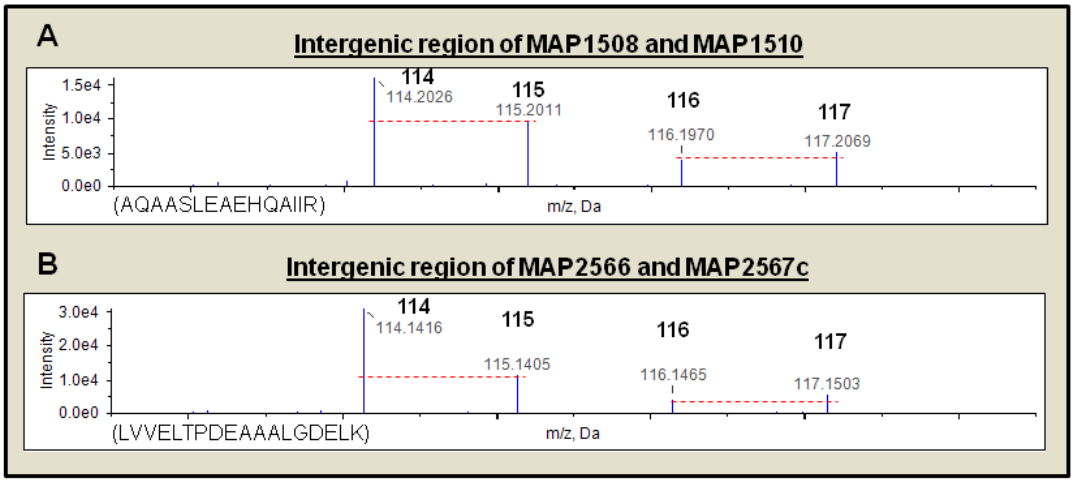


Figure 16: Reporter ion regions (114 – 117 *m/z*) of peptide tandem mass spectrum from iTRAQ labeled peptides from unannotated genomic regions of MAP. Quantitation of peptides and inferred proteins are made from relative peak areas of reporter ions. Peptides obtained from type II MAP cultures grown in iron-replete and iron-limiting medium were labeled with 114 and 115 reporter ions, respectively.. Peptides obtained from sheep MAP cultures grown in iron-replete and iron-limiting medium were labeled with 116 and 117 reporter ions, respectively. The peptide sequences and shown in the parenthesis and the red dashed line illustrates the reporter ion relative peak intensities. Both the peptides were upregulated by type II MAP strain under iron-replete conditions.

Figure 17: Iron dependent metabolic programming in cattle and sheep MAP

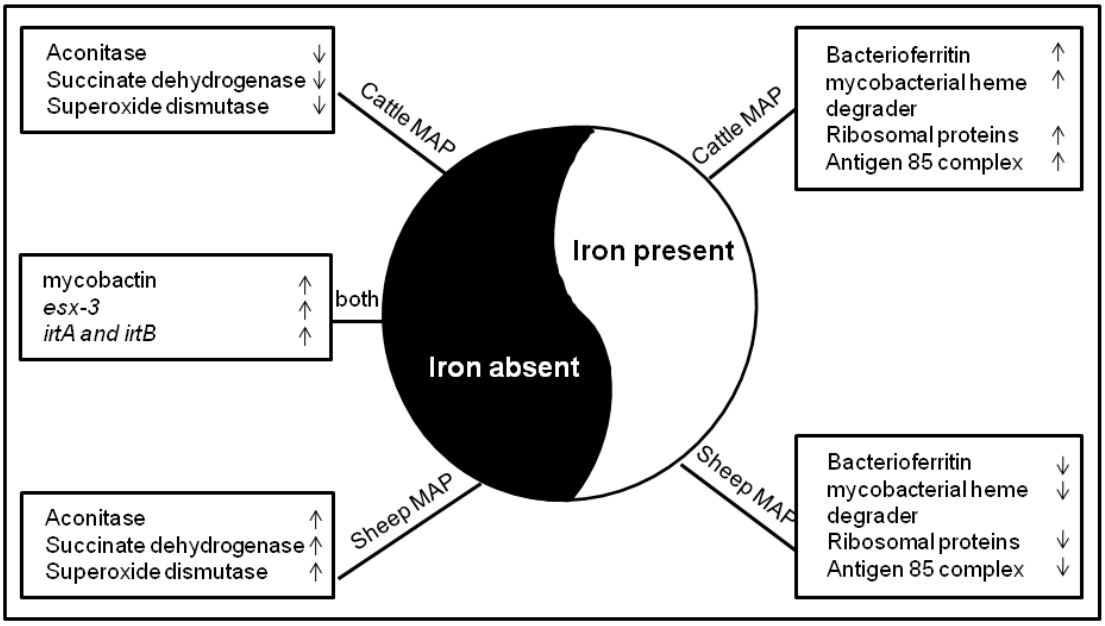


Figure 17: Under iron-replete conditions, there is upregulation of ribosomal proteins, bacterioferritin, mycobacterial heme, utilization and degrader proteins in cattle strain (type II) alone. Under iron limiting conditions, siderophore synthesis and transport genes are upregulated in both type I and type II MAP strains. However, under iron limitation there is downregulation of aconitase, succinate dehydrogenases and superoxide dismutase in type I strain alone. This suggests an iron-sparing response exclusively in type II but not type I strain.

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CHAPTER 5: PRIMARY TRANSCRIPTOMES OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* REVEAL PROPRIETARY PATHWAYS IN TISSUE AND MACROPHAGES

Mycobacterium avium subsp. *paratuberculosis* (MAP) persistently infects intestines and mesenteric lymph nodes leading to a prolonged subclinical disease. MAP genome sequence was published in 2005, yet its transcriptional organization in natural infection is unknown. While the prior research analyzed regulated gene sets utilizing defined, in vitro stress related or advanced surgical methods with various animal species, we investigated the intracellular lifestyle of MAP in the intestines and lymph nodes to understand the MAP pathways that function to govern this persistence. Our transcriptional analysis shows that 21%, 8% and 3% of the entire MAP genome was represented either inside tissues, macrophages or both, respectively. Transcripts belonging to latency and cell envelope biogenesis were upregulated in the intestinal tissues whereas those belonging to intracellular trafficking and secretion were upregulated inside the macrophages. Transcriptomes of natural infection and in vitro macrophage infection shared genes involved in transcription and inorganic ion transport and metabolism. MAP specific genes within large sequence polymorphisms of ancestral *M. avium* complex were downregulated exclusively in natural infection. We have unveiled common and unique MAP pathways associated with dormancy, cell wall biogenesis, iron metabolism and virulence in naturally infected cow intestines, lymph nodes and in vitro infected macrophages. This dichotomy also suggests that in vitro macrophage models may be

insufficient in providing accurate information on the events that transpire during natural infection. This is the first report to examine the primary transcriptome of MAP at the local infection site (i.e. intestinal tissue). Regulatory pathways that govern the life cycle of MAP appear to be specified by tissue and cell type. While tissues show a "shut-down" of major MAP metabolic genes, infected macrophages upregulate several MAP specific genes along with a putative pathogenicity island responsible for iron acquisition. Many of these regulatory pathways rely on the advanced interplay of host and pathogen and, in order to decipher their message, an interactome must be established using a systems biology approach. Identified MAP pathways place current research into direct alignment to meeting the future challenge of creating a MAP-host interactome.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is one of the most well documented persistent infections of ruminants worldwide (Johne's disease (JD)) and yet the cues leading to its intracellular survival live in obscurity (19). Major hindrances involved in examining gene regulation during MAP infection are the low amounts of bacterial RNA isolated from an infected host and the lack of an appropriate animal model (34). In order to overcome the limited quantity of RNA, previous transcriptomic studies interrogating genes used in pathogenic mycobacterial infection were conducted utilizing mimetic conditions of infection in an in vitro environment (i.e. hypoxia, nutrient starvation, acid and nitric oxide (NO) stresses, etc.) (34, 45).

While these studies provided insight into a finite number of genes regulated by specific cues, it is not representative of natural infection since mycobacteria will encounter more than one stress at a time. Therefore we hypothesized that the transcriptional organization of MAP in lesions of naturally infected animals as well as in vitro infected macrophages, we will be able to understand the pathogenic lifestyle of MAP.

Multiple stressors may change which genes are utilized as well as potential for gene:gene or protein:protein interactions that influence survival and dissemination in the host. Therefore, current investigations into the intracellular fate of MAP and host responses rely on in vitro macrophage models, specifically bovine and murine cells (21, 29, 40-42). Studies from our laboratory using an in vitro bovine macrophage infection model in conjugation with selective capture of transcribed sequences (SCOTS) revealed upregulation of MAP genes involved in combating oxidative stress, metabolic and nutritional starvation and cell survival at 48 and 120 h post infection (47). These results indicate that common sets of genes are required for MAP to persist within a multifaceted host environment. Furthermore, consistent with another study using SCOTS analysis with *Mycobacterium avium*, MAP expresses several genes involved in fatty acid degradation, which has been suggested as a universal theme used by pathogenic mycobacteria to successfully efface and invade macrophages and other cell types (20, 27).

More importantly, in vitro macrophage studies do not address the initial events that set the venue for MAP's transition into the macrophage. Prior to residing inside

intestinal macrophages, MAP must first encounter the intestinal epithelium (19). The intestinal epithelium represents a formidable fortress that actively secretes IgA and antimicrobial peptides, which is shielded by the glycocalyx and a thick layer of mucus, produced by intestinal goblet cells (7). Therefore, it is of little surprise that most of the disease signs associated with JD (i.e. transmural inflammation, corrugation, and gross lesions) are inflicted upon the intestinal tissue. Despite MAP's successful siege against the intestinal barrier as evidence of its infiltration into lamina propria macrophages, the exact genes and pathways MAP employs within the intestinal epithelium remains a black hole in our understanding of overall pathogenesis (3). Furthermore, it has been suggested that MAP processing by the epithelium may aid in efficiency of invasion in macrophages by pre-exposure to a hyperosmolar environment or expression of a MAP oxidoreductase (MAP3464) (3, 31). Thus, it seems short-sighted to assume that no disparate mechanisms are used to survive in the intestinal tissue and macrophage given two different cell types with varying function.

METHODS

All cattle work in this study was performed according to institutional guidelines and approved animal care and use protocols at the University of Minnesota.

Sampling from subclinical JD cows: Two sub-clinically infected but apparently healthy dairy cows, identified as low shedders by routine serological and fecal culture methods at the University of Minnesota Veterinary Diagnostic Laboratory, were purchased from a

farmer and euthanized for this study. The infection status of the animals was established using standard serology for MAP-specific antibody (Idexx Laboratories, Inc., Westbrook, ME) (9) and fecal culture (12). At necropsy, sections from affected portions of the intestines ileum, ileocecal junctions, and the surrounding enlarged mesenteric lymph nodes were harvested and either snap-frozen in liquid nitrogen or fixed in formalin for RNA extraction and histopathological examination, respectively. Organs were triturated and cultured for of the presence MAP using standard mycobacterial culture techniques.

Genotyping of MAP: MAP colonies were sub-cultured in Middlebrook 7H9 broth (MB7H9) (DIFCO, Lawrence, KS) supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Fischer Scientific, Inc., Pittsburgh, PA) and mycobactin J (2 mg/L) (Allied Monitor, Inc., Fayette, Missouri) at 37^{o}C with subtle shaking. MAP isolates were determined free of contaminant bacteria by absence of growth on Brain-Heart Infusion (BHI) agar at 37^{o}C. Following genomic DNA extraction using a standardized protocol (Qiagen, Valencia, CA), isolates were confirmed for MAP specific IS*900* insertion sequence by PCR and agarose gel electrophoresis. MAP isolates from infected tissues as well as MAP cattle strain K-10 (MAP K-10) were genotyped based on short sequence repeats (SSR) from two polymorphic (G and GGT) loci as described (4, 30).

Macrophage infection assay: Monocyte derived macrophages (MDMs) were prepared from fresh peripheral blood mononuclear cells obtained from the jugular vein of a JD-

free healthy cow and grown in 25cm^2 flasks (~2.0 x 10^7 cells/flask) at 37^{o}C in 5% CO_2 as stipulated by a previously described method (21, 48). A seed stock of MAP K-10 was sub-cultured and grown to mid-logarithmic growth phase (OD_{600} = 1.0) in MB7H9 broth (supplemented with OADC enrichment medium and 2 µg/ml of mycobactin J) at 37°C on a shaker set at 120 rpm. MAP K-10 was used at a 20:1 multiplicity of infection (MOI) in all infections. Following infection after two hours, MDMs were washed twice with fresh, pre-warmed serum-free RPMI 1640 (Gibco® Invitrogen, Inc., Carlsbad, CA) to remove non-adherent bacteria and the cultures were subsequently grown in RPMI 1640 with 2% autologous serum for 6, 48 and 120 hrs in duplicate at each time point.

Nucleic acid extraction: For total RNA extraction, ~30 mg mesenteric lymph nodes and ileum were ground separately in liquid nitrogen using a mortar and pestle and dissolved in 600 µL of RLT buffer (Qiagen Inc., Valencia, CA). Total RNA from infected MDMs (6, 48 and 120 hrs p.i,) and MAP K-10 broth cultures were extracted by TRIzol reagent (Invitrogen Inc., Carlsbad, CA) per manufacturer's instructions. Samples were homogenized in a mini bead-beater (Biospec) with 0.3ml of 0.1mm sterile RNase-free zirconium beads for 4 min. followed by RNA extraction using RNeasy (Mini) kit (Qiagen Inc., Valencia, CA). All samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to eliminate genomic DNA contamination. The purity and yield of total RNA samples was examined using Nanodrop spectrophotometer (Thermo Scientific Inc., Wilmington, DE) and Agilent 2100E Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All samples were stored at -80°C until later analysis.

Sample processing and microarray hybridizations: All microarray experiments were conducted using the minimal information about a microarray experiment (MIAME) guidelines. Polyadenylated host mRNA and bacterial rRNA were eliminated by processing the samples with MICROB*Enrich* and MICROB*Express* Bacterial mRNA Purification Kits (Ambion Inc., Austin, TX), respectively. RNA samples were amplified using MessageAmp™ II-Bacteria Kit for prokaryotic RNA amplification system (Ambion Inc., Austin, TX) and labeled with SuperScript™ Plus Direct cDNA Labeling System (Invitrogen Inc., Carlsbad, CA). MAP transcripts from infected tissues (two sections each for ileum and mesenteric lymph node) and macrophage infection assay (performed in duplicates) were combined individually with sheared genomic DNA of MAP K-10 labeled with BioPrime® Plus Array CGH Genomic Labeling System (Invitrogen Inc., Carlsbad, CA) and hybridized onto 70-mer oligonucleotide microarrays (obtained from Dr. Michael Paustian, NADC, Iowa). Every predicted open reading frame in the MAP strain K-10 genome is represented on this array. One 70-mer was designed for each gene with a total length of less than 4000 bp, while longer genes were split in half and one 70-mer oligo was designed for each half. Additional details of this microarray design can be found elsewhere (32). RNA from MAP K-10 broth culture and tissue isolates was also processed in the same manner. After overnight hybridization, microarray slides were washed and scanned using the HP Scanarray 5000 (PerkinElmer Inc., Waltham, MA). Images were collected and stored for expression analyses.

Microarray data analysis: Numeric data was extracted from the two-channel hybridization images using the microarray image analysis software, BlueFuse (BlueGnome Ltd, Cambridge). Following normalization by global lowess, the gene expression data was analyzed by GeneSpring GX 10.0 (Agilent Technologies Inc., Foster city, CA). Two group T test was performed to identify the differentially expressed MAP genes (DEGs) and multiple test correction was applied to the T test. The DEGs in natural infected tissues (ileum and mesenteric lymph nodes) and in vitro infected macrophages were identified after normalizing the data with MAP in broth culture. The lists of genes obtained from the above were analyzed using Basic Local Alignment Search Tool (BLAST) algorithm in National Center for Biotechnology Information (NCBI) database against the MAP K-10 genome and the 11 mycobacterial genomes listed in the NCBI databank. Gene IDs were categorized into various functional groups based on Clusters of Orthologous Groups (COGs). Differentially regulated genes were also uploaded in Pathway Studio 6.0 (Ariadne genomics Inc., Rockville, MD) with the *M. tuberculosis* H37Rv database to explore the cellular context of differentially expressed genes by computational methods of protein network identification.

Quantitative Real-time PCR validation: Selected genes from microarray data were validated using two-step SYBR-green based quantitative real-time PCR (Roche, Indianapolis, IN) analysis in Roche LightCycler 480 II (Roche Inc., Indianapolis, IN). Primers were designed using web-based tools, Primer3 (http://frodo.wi.mit.edu/primer3/) or Universal Probe Library (Roche Inc., Indianapolis, IN) and verified by BLAST

searches to confirm their specific binding to target sequences (Table 6). The following cycle program was used: denaturation at 95^{o}C for 15 min. and PCR at 95^{o}C for 10 s, 65^{o}C for 15 s, 72^{o}C for 22 s for 55 cycles. Test and control samples were normalized using the house keeping gene, *secA*, and relative expression was calculated by $2^{-\Delta\Delta CT}$ method (24).

RESULTS

Isolation and identification of MAP: Postmortem examination of two subclinical JD cows revealed gross lesions and corrugation throughout the intestine indicative of chronic inflammation, especially within the ileum (Fig. 18 A is a representative example). Histopathological sections of the ileum identified MAP by modified Ziehl-Neelson staining for acid-fast organisms (Fig.18 B), which was later confirmed by standard culture and PCR methods (Table 7). MAP was successfully isolated from intestinal lesion, mesenteric lymph nodes, liver and spleen of both subclinically infected animals. All isolates were genotyped by SSR analysis as >13G and 5GGT repeats, which was identical to MAP K-10 culture (15G and 5GGT) used for macrophage infection.

Enrichment and confirmation of MAP transcripts: Total RNA obtained from naturally infected tissues and experimentally infected MDMs were processed to remove host RNA as well as ribosomal RNA. Similarly, the total RNA from broth cultures of tissue isolates and MAP K-10 were enriched for bacterial messenger RNA by removing

ribosomal RNA. All samples were subjected to RNA amplification and analyzed on a regular denaturing agarose gel and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Furthermore, the presence of MAP specific genes was confirmed using RT-PCR, sequencing and BLAST analysis (data not shown) prior to use in microarrays.

Gene expression of MAP during natural infection: Analysis of MAP from infected tissues showed differential expression of 2167 genes compared to broth cultures. After multiple test correction, 1795 genes were significantly different at $q \leq 0.05$ by unpaired t-test. Amongst these, 1684 genes were altered at ≥ 1.5 fold change and 1054 genes at fold change ≥ 2.0 compared with corresponding MAP isolates in broth culture. Genes belonged to a variety of functional categories such as mammalian cell entry (MCE family proteins), antigen variation (PPE family proteins), pH regulation and immunogenicity (serine/threonine protein kinases), DNA replication (transcriptional regulators), cell envelope biogenesis (integral membrane proteins, major membrane proteins), ion transport (ABC transporters), energy production and conversion (oxidoreductases, ATPases, methyl transferases), carbohydrate metabolism (glucose-6-phosphates, glucokinases), fatty acid metabolism (acyl-coA dehydrogenases, glycosyl transferase), enzyme biosynthesis for amino acid transport and latency associated transcripts. Table 8 shows a list of operons and Table S12-S14 shows complete lists of genes differentially regulated during natural infection.

Shared and variable genes between the ileum and MLN are represented in Table S12-S14. Genes were classified into various functional groups based on clusters of

orthologous genes (COG) classification and the percent gene expression of each group was calculated. Functional groups enriched in both ileum and MLN belonged to defense mechanisms (i.e. MAP1575c, MAP3162c), unknown function or poorly described cellular pathways (i.e. MAP3812c, MAP4269c). Genes belonging to transcription (i.e.: MAP1736, MAP2418) and lipid metabolism and transport (i.e.: MAP0556c, MAP1451) were specifically enriched in the ileum, while energy production and conversion (i.e.: MAP1171, MAP2620c) and inorganic ion transport and metabolism (i.e.: MAP0982c, MAP3141c) were enriched in MLN (Fig. 19).

Gene expression of MAP in an in vitro macrophage infection assay: A total of 562 MAP genes were differentially expressed during macrophage infection compared with broth culture. Amongst them, 556 genes had a ≥1.5 fold change and 462 genes had ≥2.0 fold change ($p \leq 0.05$). At 6 hr post infection (PI), upregulated genes of significant interest included serine/threonine protein kinase, *pkn*B (MAP0016c), ATPase, AAA family protein (MAP0167) and PPE family protein (MAP1675). At 48 hour PI MAP upregulated PE family proteins (MAP0140; MAP0339, MAP1507), transcriptional regulators (MAP0475; MAP2428c) and *fad*D27 protein (MAP3156). Finally, at 120 hr PI MAP displayed higher induction of genes concerning major membrane protein, *mmp*L4 (MAP0076, MAP1240c), MCE-family proteins (MAP0566, MAP0759), PE-family proteins (MAP0140; MAP4076), oxidoreductase (MAP0444; MAP3507), lipase, *lip*E (MAP0248) and ABC transporters (MAP0563). A total of 55 genes were shared across different time points in the macrophage infection assay using MAP K-10 strain. Fig. 19

shows the distribution of the differentially expressed genes across three time points and Table S15-S17 shows the detailed list of genes.

Comparisons of gene expression profiles of naturally infected tissues and in vitro macrophage infection: While a total of 126 genes were commonly expressed between infected tissues and macrophages, 928 and 336 genes were specifically represented in tissues or macrophages, respectively (Fig. 19, 20 and Table S18). Functional categories belonging to transcription (MAP1631c, MAP1634, MAP3967) and inorganic ion transport and metabolism (MAP1110, MAP3773c, MAP4171) were represented both in tissues and macrophages. Macrophage specific gene expression represented functional categories belonging to cell cycle control (MAP2990c), cell wall biogenesis (MAP0670c), cell motility (MAP1506) and secretion (MAP1515). Tissue specific gene expression included genes categorized into defense mechanisms and those that were not represented in any of the COG groups. Furthermore, MAP regulates expression of latency related genes such as MAP0033c (WhiB family protein), MAP0038 (probable biofilm regulator), and MAP0075 (mycobacterium specific membrane protein) during natural infection.

Expression of MAP lineage specific genes during natural infection: Approximately 96 genes distributed in six loci (LSP 4, 11, 12, 14 and 15) were recently described as MAP lineage specific genomic insertions; majority of these genes were consistently upregulated (fold change > 2.0, $p < 0.05$) in the in vitro infected macrophages whereas downregulated in the tissues of both the animals (Table S19 and S20) (1). Loci of interest include LSP 4 and 11, which carry putative prophages, transposons and unique sequences

with no hits in NCBI. MAP0858, located within LSP 4, has conserved domains resembling those of a virulence factor (proteophosphoglycan) belonging to *Leishmania.* LSP11 contains MAP2149c, which has conserved domains to that of SARP (**S**treptomyces **A**ntibiotic **R**egulatory **P**rotein) family transcriptional factor. Located within LSP 12 is a mammalian cell entry (*mce*) operon (MAP2190 – MAP2194) which was downregulated in the tissues whereas MAP2189 (*mce*) and MAP2180c (a beta lactamase like protein) were upregulated in the macrophages. An enzyme involved in xenobiotic biodegradation and metabolism (MAP1728c *yfnB*) was downregulated in the tissues whereas upregulated in the in vitro infected macrophages.

Expression of iron acquisition genes during natural and in vitro macrophage infection: We identified an increased expression of genes involved in iron metabolism inside the tissues and macrophages. These included mycobactin biosynthesis genes, ABC transporters and genes belonging to *esx-3* secretion system. Furthermore, iron storage gene, *bfrA,* was downregulated. Interestingly, we identified the expression of a pathogenicity island (LSP14) present exclusively in MAP. Bioinformatic analysis indicated that LSP14 may play a role in maintaining intracellular iron homeostasis in MAP. Genes located within LSP14 belong to ABC transporter operon (MAP3731c - MAP3736c), siderophore biosynthesis operon (MAP3741 - MAP3746) and oxidoreductase (MAP3756c). The oxidoreductase (MAP3744), and ABC type transporter (MAP3739c) and a polyketides synthase (MAP3763c) belonging to LSP14 were all upregulated in macrophage infections. An ABC transporter operon (MAP3774c -

MAP3776c), which is located on LSP 15, was downregulated in infected tissues alone. MAP3773c, a probable Ferric Uptake Regulator protein on LSP 15, was downregulated in the tissues and upregulated in experimentally infected macrophages.

Real-time PCR based validation of microarray data: We selected seven genes for real-time PCR for validation of microarray results. These genes are chosen based on their roles in diverse pathways. Selected genes included membrane protein (MAP0283c), inorganic ion transport (MAP0782, MAP2488), iron acquisition (MAP2173c), energy production and metabolism (MAP3898, MAP4120) and finally an LSP specific for cattle strains of MAP (MAP1728c). RNA extracts used for microarray analysis were also analyzed for their level of expression by real-time PCR assay with primers designed using universal probe library (Roche, Indianapolis, IN). The expression of these genes in the tissues of JD cows shows the same trend in microarray and the real-time analyses. Fig. 21 demonstrates the fold change ratios of selected MAP genes in the microarrays as compared to their gene expression in real-time after normalization with a housekeeping gene *secA*.

DISCUSSION

The hallmark of MAP infection is the subclinical manifestation of a persistent intestinal infection. Yet, surprisingly, there remains a paucity of studies investigating the intracellular lifestyle of MAP in the intestinal epithelium in comparison to research involving macrophage and/or lymphocyte models (21, 48). We sought to fill this critical

knowledge gap by reporting the first transcriptome analysis of MAP in infected tissues and macrophages. Both the ileum and mesenteric lymph node have been suggested to act as potential MAP reservoirs within the host; therefore, it is critical to understand the MAP pathways that function to govern this persistence (11, 19, 44). The current trend in MAP research is to isolate and analyze regulated gene sets given defined, in vitro stress related cues or during a particular infection stage using surgical methods and various animal species (2, 23, 45). However, we have taken a more directed approach to uncover common and unique pathways utilized by MAP in intestinal tissues using the natural host under natural infection. Elucidation of the transcriptome active in local infection sites is expected to augment our knowledge of MAP pathogenesis such as mechanisms involved in maintaining intracellular iron homeostasis of MAP. Furthermore this study will lend itself to the establishment of a host-pathogen interactome, as well as rational design of vaccines and/or antimycobacterial therapeutic modalities.

MAP residing within the ileum is primed to enter dormancy in subclinical infection:

The uncanny ability of pathogenic mycobacteria to persist within the host for an indefinite period of time that can last several years is intimately linked with dormancy (10, 46). Although the genes and signals that induce dormancy remain unclear, mycobacteria entering this phase are characterized by a state of chronic or prolonged non-replication (46). Our results show responses to iron deprivation by MAP – a response that is possible used by the organism to maintain a minimal level of metabolic activity during chronic infection.

One cue that primes the cell to enter into the non-replication stage is the stringent response, which is characterized by the *relA* controlled production of hyperphosphorylated guanosine ((p)ppGpp) activated upon nutrient deprivation, hypoxia and oxidative stress (6, 33). Together *relA* and (p)ppGpp is able to combat hostile environments by negatively regulating bacterial "life" signals such as DNA and protein machinery. Interestingly, we have identified a unifying theme from naturally infected host tissue as the downregulation of several energy, carbohydrate, amino acid and lipid metabolism as well as transcriptional and DNA replication related genes. Similar attributes of the stringent response were found to be selectively upregulated within the ileum. For example, the RelA/SpoT domain-containing protein has a three-fold upregulation in the ileum. Recently Geiger and colleagues have shown that the RelA/SpoT domain-containing protein, RSH synthetase/hydrolase enzyme, in *Staphylococcus aureus* is responsible for maintained production of (p)ppGpp and concomitant repression of genes regulating translational machinery (16). Furthermore, a single metabolism gene regulating menaquinone biosynthesis and consequent production of vitamin K (MAP4052) was uniquely upregulated in the ileum (5). In addition to initiating the synthesis of mycobactins, menaquinone biosynthesis genes have been shown to be a critical factor in maintaining non-replicating mycobacterial cell viability (14).

Stringent response priming of MAP cells is most likely due to host inflicted stresses, particularly nitric oxide resultant in DNA damage (6). Previous studies examining MAP "scrapings" from the intestinal wall of JD clinical cattle show significant

upregulation of Fur regulated *katG*, a bacterial catalase-peroxidase gene used to combat oxidative stress (17). Furthermore, granulomatous lesions within the ileum or lymph node isolated from cattle naturally infected with either MAP or *M. bovis*, respectively, have enhanced immune-staining for natural resistance-associated macrophage protein 1 (NRAMP1) and inducible nitric oxide synthase (iNOS), which together synthesize nitric oxide (13, 15). Although we did not identify enrichment of *katG* in the ileum, we show upregulation of MAP2836, a LexA repressor, which is stimulated upon DNA damage and stress and results in the arrest of cell division and induction of DNA repair (8). Similarly, increase in a LysR transcriptional regulator (MAP2442) within the ileum is indicative of an oxidative stress response (25). These data suggest that during the early stages of infection, MAP is primed to enter dormancy by the stringent response in order to avoid oxidative stress and DNA damage. This appears to be a "watershed moment" in the intracellular lifecycle of MAP as dormancy during subclinical infection will ensure its survival and future dissemination into other organs.

MAP evades immune detection in the MLN by forming a "Roman Turtle": Similar to MAP pathways found in the ileum, the majority of MAP genes involved in energy, carbohydrate, inorganic ion including iron, DNA repair, transcription and translation pathways are downregulated. However, there is a lack of stringent response as well as latency-associated expression.

The MLN contains populations of circulating effector cells, such as T and B cells; therefore, MAP may downregulate the aforementioned pathways to avoid detection by

the host immune system (7). Furthermore, common to both ileum and MLN, MAP upregulates several genes associated with mycobactin storage, cell envelope and outer member biogenesis (MAP1905c, MAP3019c and MAP3979). It is well established that mycobacterial cell wall components have immunomodulatory functions that enable pathogenic mycobacteria to escape immune surveillance by suppression of pro-inflammatory cytokines, phagosome-lysosome fusion and MHC class II presentation (28, 35, 38, 40). Thus, MAP may establish a microbial "Roman Turtle" tactic to surround itself with complex cell wall associated glycolipids to prevent recognition and persist unabated by the host immune system.

Expression of MAP unique pathogenicity island involved in iron acquisition during natural and in vitro macrophage infection: Comparative genomics of the *M. avium* complex (MAC) revealed that MAP evolved as a pathogen by acquiring large segments of DNA (i.e. pathogenicity islands) via horizontal gene transfer (1, 26, 39, 43). Our study is the first to directly show that some of these putative pathogenicity islands are associated with virulence. Contrary to expression found within the tissues, genes belonging to the LSPs were upregulated in macrophage infection. Qt-RT-PCR analysis also demonstrated that MAP1728c (*yfnB*), a gene involved in xenobiotic biodegradation and metabolism located within the LSP (deletion 2) specific for cattle MAP strains (1) was downregulated in the tissues. This is consistent with the regulation of other MAP genes, which suggests that MAP transcriptional machinery remains silent in the tissues. Several iron related genes were downregulated in tissues including LSP15, a MAP

unique pathogenicity island that encodes a ferric uptake regulator (MAP3773c), as well as the ABC transporter (MAP3731c - MAP3736c), and a possible siderophore biosynthesis operon (MAP3741-MAP3746) that contains a FUR binding box within the intergenic region (Fig 22) (37). This is of significant interest as part of this region (MAP3731c-MAP3736c) has been previously shown to be immunogenic and preliminary studies indicate its use as a potential vaccine candidate (18). Furthermore, our genome analysis revealed that a type VII secretory system (esx3) was located immediately downstream of LSP15. Esx3 has recently been shown to be essential for mycobactin synthesis (36) and we have identified its repression by the MAP iron dependent regulator (IdeR) in the presence of iron (22). Taken together, our functional analysis suggests that LSP14, 15 and esx-3 form a major pathogenicity island that plays a potential role in maintaining iron homeostasis and hence survival and/or persistence inside the host.

TABLE 6: Primer sequences used in Q-RT PCR

Gene and direction	Sequence
MAP0233c, F	ggggtagaaggacaggaagc
MAP0233c, R	agttctacgccagcatcgac
MAP0283, F	caatcttccgggtctaccac
MAP0283, R	gagccggtactgatggtga
MAP0782, F	ttcgtgtgcctgtgcaac
MAP0782, R	gcgacttcgttggtggtc
MAP1728, F	cagccacaaatacgacatcc
MAP1728, R	gtgacgaaggctgtttgga
MAP2173c, F	gcagggtgcggtagtgac
MAP2173c, R	ccgagtatctggtcgaggtg
MAP2488, F	gccggttgctcaactacct
MAP2488, R	tcaggcagaacgtcaggaa
MAP3698, F	ccgtcgatgtaccaccagt
MAP3698,R	catcggctccttggtgat
MAP4120c, F	ggaaaccaagggatgtcgt
MAP4120c, R	acgagacgctgcaagagc
secA, F	ggcctgctccttgaggtt
secA, R	gcgcaaggtgatctacgc

TABLE 7: Fecal culture results of MAP isolated from intestinal tissues

Animal ID	Organ	Colony count	Test result	classification
386	Ileum	>100	positive	high shedder
386	Mesenteric lymph node	>100	positive	high shedder
39	Ileum	1-10	positive	low shedder
39	Mesenteric lymph node	>100	positive	high shedder

MAP organisms were grown in Herrold's egg yolk medium for 12 weeks at 37^{o}C. The colonies were counted and the animals were classified as high or low shedders as described in materials and methods.

TABLE 8: List of operons expressed in the tissues

Operon	Function
MAP0150c-MAP0152c	Acetyl-coA dehydrogenase
MAP0232c-MAP0237c	Call wall biosynthesis
MAP0564-MAP0569	MCE family
MAP1778c-MAP1780c	Lipid metabolism
MAP0107-MAP0116	MCE family
MAP2171c-MAP2177c	Mycobactin biosynthesis
MAP3464-MAP3465	ABC transporters
MAP2310c-MAP2314c	Fatty acid degradation
MAP1712-MAP1716	Fatty acid biosynthesis
MAP1522-MAP1523	PPE family
MAP2569c-MAP2571c	Glycosyl transferase

Figure 18: MAP infection in subclinically infected animals

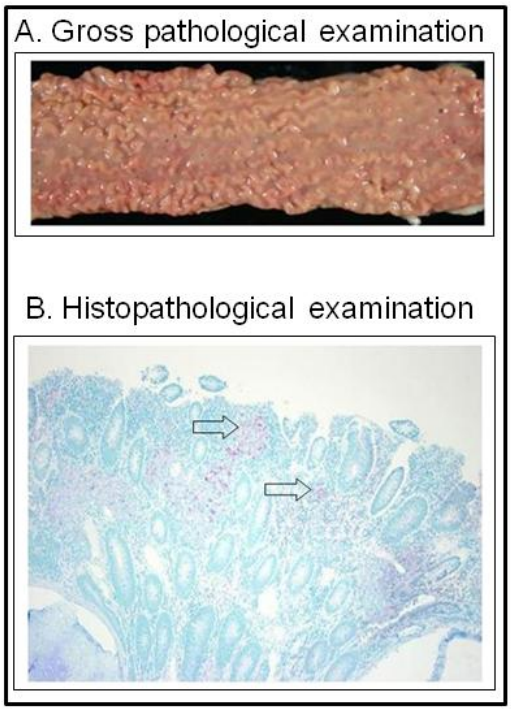

Figure 18: (A) Section of bovine ileum infected with MAP: Longitudinal section of ileum showing inflammation and corrugated appearance of inner mucosal layer from a dairy cow with subclinical Johne's disease.(B) Histopathology of bovine ileum with MAP: Acid fast staining of an ileal section of subclinical JD cow in Fig.1A showing MAP organisms (100x).

Figure 19: Classification of differentially expressed MAP genes into Clusters of orthologous genes (COG) groups

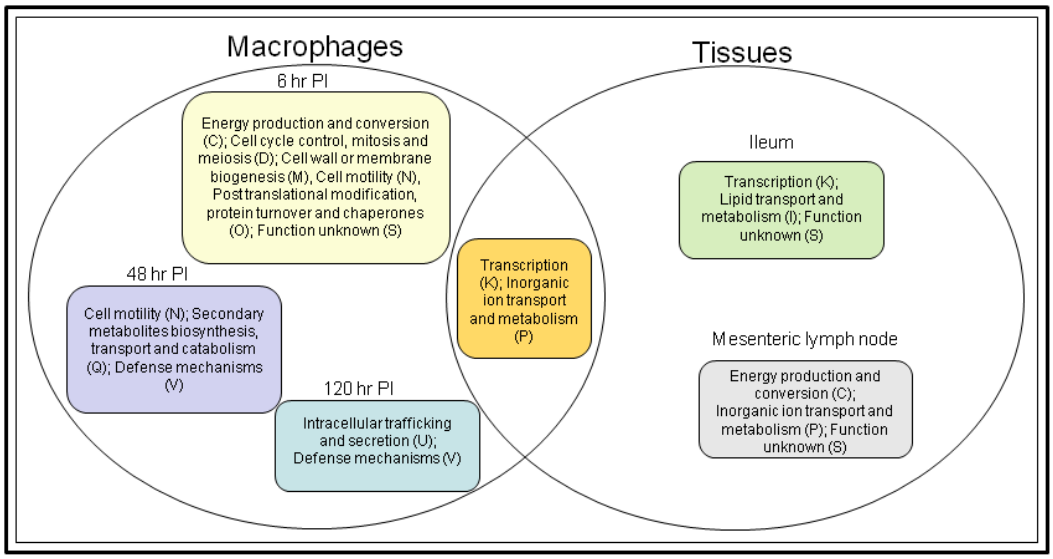

Figure 19: Differentially expressed genes in the tissues or infected macrophages were grouped based on clusters of orthologous genes (COG) classification. Significantly enriched COGs under each condition are represented in the Venn diagram. Shown in the parenthesis is the code for each COG category.

Figure 20: Pathway analysis of COGs enriched in tissues and macrophages

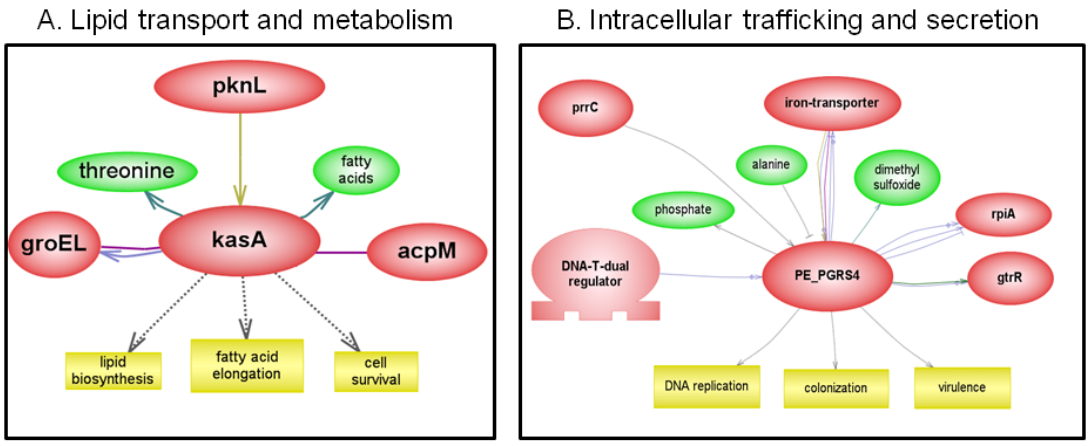

Figure 20: COGs enriched in tissues or macrophages were used to identify interactions with other groups and their diverse roles in various cellular processes using Pathway Studio 6.0 (Ariadne genomics Inc., Rockville, MD). Pictorial representation of the interactions of (A) Lipid metabolism genes centered on *kasA*, a cell wall biogenesis gene upregulated in the tissues and (B) Intracellular trafficking and secretion genes centered on PE_PGRS4, a PPE family gene upregulated in macrophages. KasA interacts with other proteins such as pknL and plays a role in lipid metabolism and cell survival. PE_PGRS4 interacts with other proteins such as PrrC, RpiA and plays a role in colonization and virulence. Green ovals indicate metabolites, red ovals indicate genes and gold rectangles indicate processes.

Figure 21: Comparisons of fold changes of selected genes by microarray and real time RT PCR

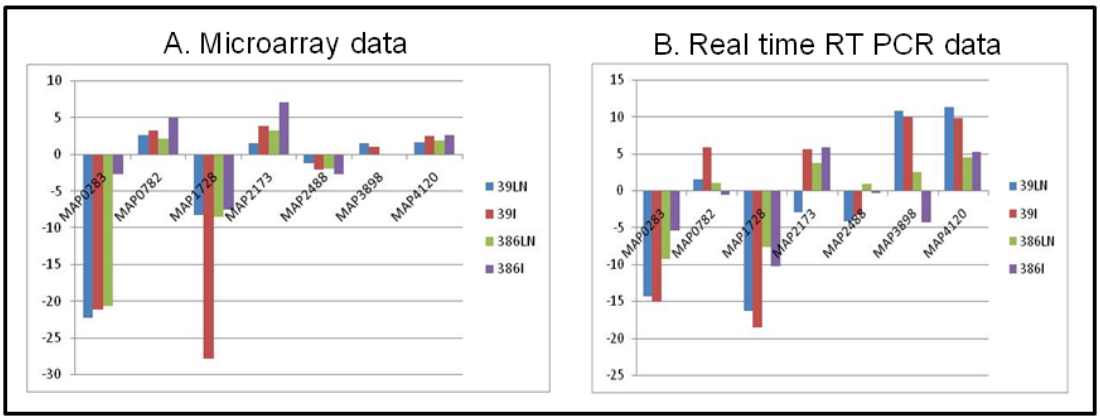

Figure 21: (A) Selected MAP genes that were differentially regulated (up or down) after subtraction with broth culture (data in linear scale). (B) These genes were validated for their expression pattern by real-time PCR to demonstrate similar trends in gene expression (data in logarithmic scale).

Figure 22: Regulation of putative iron acquisition pathogenicity island during natural infection

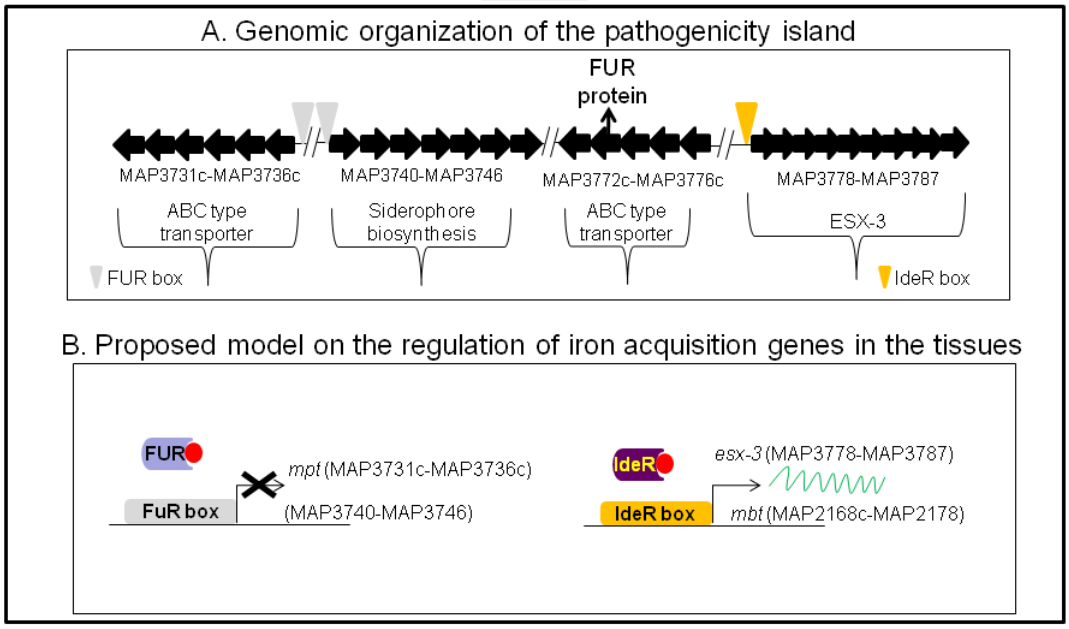

Figure 22(A) Genomic organization of a MAP specific pathogenicity island differentially expressed in the tissues. ABC transporter (*mpt*) and siderophore biosynthesis operons were downregulated whereas *esx-3* and mycobactin synthesis (*mbt*) operon (not shown) were upregulated in the tissues. *mpt* operon is immunogenic and *esx-3* is involved in transporting ferrated mycobactin into the cell. (B) Proposed model for FuR and IdeR mediated regulation of iron acquisition genes. IdeR derepresses the synthesis of *mbt* and *esx-3*. FUR represses expression of putative iron acquisition genes probably because they are immunogenic. IdeR regulation is well characterized in MAP whereas FUR regulation needs to be tested.

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CHAPTER 6: CONCLUSIONS

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD) in cattle, requires a siderophore (mycobactin) supplementation for optimal growth in laboratory media. This unique iron requirement makes MAP fastidious often requiring eight to sixteen weeks to see colonies in culture – a major hurdle in the diagnosis and therefore in implementation of optimal control measures.

Over the past 4 years we have been working on iron regulation in MAP through a series of scientific experimentation we have begun to unravel the secrets of metabolic networks of this organism. Since Twort and Ingram's first description in 1912 that MAP requires a necessary "food stuff" for cultivation in vitro there has been only incremental progress in understanding its nutrient requirements (7). Experiments performed by Francis and Snow resulted in the identification of the "food stuff" as mycobactin (1). Since MAP genome sequence was published in 2005, very little research was focused on iron physiology of this fastidious organism. Understanding iron regulatory networks in MAP is essential for several reasons. First, the genome sequence suggests that a deletion in *mbtA* as a possible reason for its in vitro mycobactin requirement (5). Yet, our studies show that MAP transcribes mycobactin genes in macrophages and in the tissues of JD positive cattle suggesting that mycobactin-based iron acquisition is fully operational (4); however, MAP appears to still need the Twort and Ingram's necessary "food stuff" for in vitro culture. Second, iron regulation in bacteria is tightly controlled and is considered a

major virulence factor – an attribute that is poorly understood in MAP. Thus a comprehensive understanding of these iron regulatory networks of MAP was lacking.

Several microbiological and genotyping studies and clinical observations suggest that JD in certain hosts such as sheep, deer and bison is caused by a distinct set of strains that show a relatively high degree of host preference. At least two microbiologically distinct types of MAP have been recognized. A less readily cultivable type is the common, but not invariable, cause of paratuberculosis in sheep (type I), while another readily cultivable type is the most common cause of the disease in cattle (type II). Thus we hypothesized that iron dependent gene regulation is different between the two strain types of MAP. We provide rigorous data that support this hypothesis.

We first set out to identify and characterize the iron dependent transcriptional factor of MAP. The putative iron dependent regulator (IdeR) of MAP was annotated in the opposite orientation on the K-10 genome. We corrected the annotation and characterized the function of IdeR. We identified that the promoter polymorphisms of the iron storage gene (*bfrA*) led to repression in the presence of iron in sheep MAP strains. This is counter-intuitive, as we would expect to have upregulation of iron storage proteins under excess iron conditions in order to prevent oxidative damage. This is likely if – (a) an alternate *bfrA* promoter start sites exist in sheep MAP strains; (b) type I strains carry additional or alternate functional iron storage genes/pathways; and (c) iron concentrations necessary for derepression of *bfrA* is significantly different between cattle and sheep MAP strains.

Literature review revealed that studies addressing the optimization of MAP culture conditions showed that iron concentrations used in the medium can modulate growth rates of the two strain types of MAP, with type I MAP growing at a significantly slower rates relative to type II MAP strains (2). Thus we reasoned that the iron concentrations in the culture medium used to grow MAP may be detrimental for viability of type I strains of MAP.

Transcriptional and proteomic profiling of the two MAP strains under iron-replete or –deplete conditions revealed that type I MAP strains are metabolically inept under iron-replete conditions as evidenced by downregulating expression of several ribosomal proteins and those involved in iron storage and recycling (Figure 23). Furthermore, iron-sparing response to iron limitation was unique to the cattle MAP (type II) strain as identified by posttranscriptional repression of iron utilization proteins. The mechanisms of posttranscriptional regulation as well as their control in the two MAP strains need further evaluation. It is likely that the new generation sequencing (RNA-Seq) technologies would provide the required resolution to identify posttranscriptional regulator(s) (6).

No studies on a pathogen are complete until its lifestyle in its natural host is understood. Intracellular lifestyle of MAP in the intestines and lymph nodes of natural infection revealed that MAP deployed genes involved in maintaining iron homeostasis in the tissues of infected animals. These studies also identified the existence of a MAP specific pathogenicity island with a potential role in iron homeostasis inside the host. The pathogenicity island carries the Fur regulator and iron acquisition genes. This raises

several questions about the role and regulation of Fur in MAP that should be the focus of future studies. Understanding the role of proteins encoded on this pathogenicity island in vivo is important because some are immunodominant (3).

In summary, through rigorous experimentation we have provided baseline scientific information as foundations to perform more targeted functional analysis on iron dependent gene regulation in MAP. We have also shown that the iron dependent gene regulation is significantly different between cattle and sheep strains of MAP. More systematic studies seeking alternate transcriptional or posttranscriptional regulatory elements mediated via small RNAs is warranted to demystify iron metabolism in MAP.

The research findings presented in this thesis significantly advance our overall understanding of MAP iron physiology. These studies opened doors to other hypotheses on alternate mechanisms of iron homeostasis and possibility of posttranscriptional regulation in MAP function. This would further enhance our ability to not only understand the key virulence determinants of MAP but also provide strong scientific foundations to refine in vitro culture methods.

Figure 23: Iron dependent metabolic programming in cattle and sheep MAP

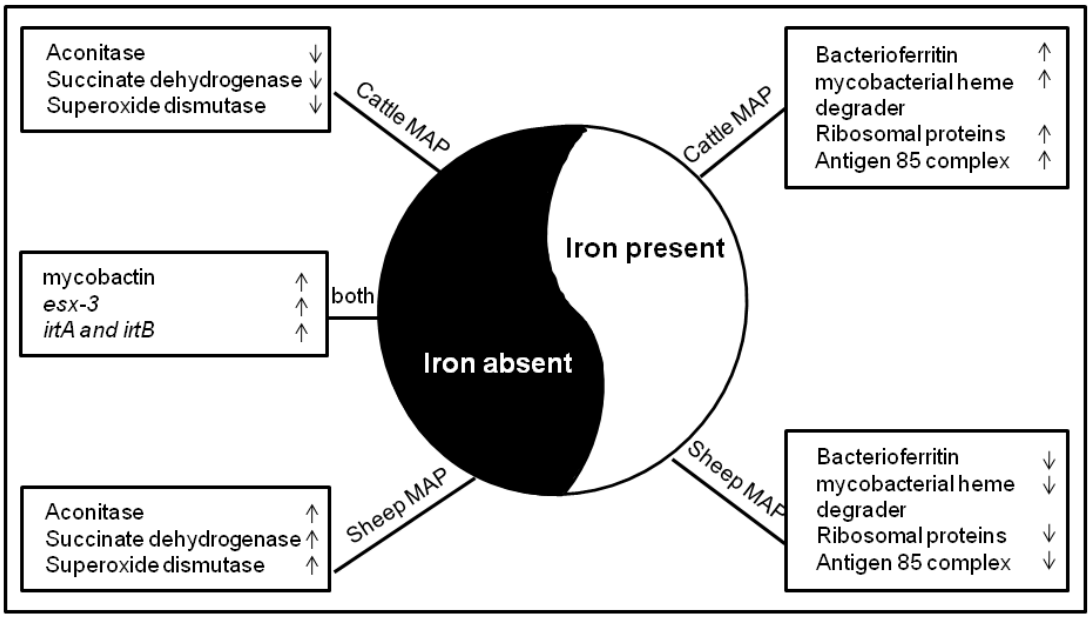

Figure 23: Under iron-replete conditions, ribosomal proteins, bacterioferritin, mycobacterial heme, utilization and degrader proteins are upregulated in cattle strain (type II) alone. Under iron limiting conditions, siderophore synthesis and transport genes are upregulated in both type I and type II MAP strains. However, under iron limitation there is downregulation of aconitase, succinate dehydrogenases and superoxide dismutase in type I strain alone suggesting an iron-sparing response operational exclusively in type II but not type I strains.

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APPENDIX

Table S1: PRODORIC predicted putative MAP IdeR regulated genes

Sl. No	Iron box sequence	ORF ID	Function
1	TTAGGTACGGCTAGCCTCA	MAP0024c	conserved hypothetical protein
2	TTCGGTATGCCTACCCTTA	MAP1551c	conserved hypothetical protein
3	TTAGGTTAGACTCCACTAA	MAP1594c	hypothetical protein
4	TGAGGTAAATCACACCTTA	MAP2816c	hypothetical protein
5	TGAGGTAAATCACACCTTA	MAP2817	hypothetical protein
6	TTAGCATTGCATAAGCTAA	MAP3778	conserved hypothetical protein
7	TAACGGTAGCCTGACCTGC	MAP3974c	conserved hypothetical protein
8	TTAGGTACGGCTAGCCTCA	MAP0025	acyl carrier protein
9	TTAGGGTTGGCTTCCCATT	MAP0184c	possible membrane transporter
10	TAAGGCTAGCGTTGCCTAA	MAP1555c	acyl carrier protein
11	TTATGTTAGCCATACAAAC	MAP2081	putative transporter protein
12	TAAGGGTTGCCTTACCAAA	MAP2414c	putative ABC transporter protein
13	GGATGCTAGGCTTACCTAA	MAP0793c	possible oxidoreductase
14	TTAGGGAAAGCTTAGGTAT	MAP2018c	FAD dependent oxidoreductase
15	TCCGGTAAGGCTCTCCGTA	MAP2648c	aldehyde dehydrogenase
16	TAACGGTAGCCTGACCTGC	MAP3975	cell envelope biogenesis
17	CTAGGCTTACCTAACGTTA	MAP0794	transcriptional regulator
18	ATAGGCAAGGCTGCCCTAA	MAP1559c	possible transcriptional regulator
19	TTAACTTAGGCTTACCTAA	MAP0193	aromatic aminoacid biosynthesis

20	TAACTTAAACATTAACTTA	MAP3069	Trans isoprenyl diphosphate synthase
21	TTAGGTTAGACTCCACTAA	MAP1595	iron storage protein
22	TTAGCGTAGCCTAGCTAAA	MAP1762c	iron permease FTR1 family protein
23	TTAGCACAGGCTGCCCTTA	MAP2177c	mycobactin synthesis
24	TTAGCACAGGCTGCCCTTA	MAP2178	mycobactin synthesis
25	ATAGGTTAGGCTACATTTA	MAP2205c	biosynthesis of siderophores

Table S2: Primers used in realtime RT-PCR

MAP ORF ID	Forward	Reverse
MAP2413c	GAGGTCGTCTTCCGTTACGA	GATCAACGCCAAAATGGTG
MAP2414c	TGTACCTCGAACAGCACCAC	CGTACCAGTTGGACCAGTCG
MAP3783	CATGTTGGACGCTCACATTC	GACTCGCCCTGGTGGAAC
MAP3784	GGATGACCTACCAGGTGTGG	CAACATGGACATGGTGTTGG
MAP1201c	AAGGTCGGCGACAAGAGTTA	CGATGTGGTCTTTGGTGATG
MAP0475	GACGATCAAAGGGGAACAAA	CTTGTCGAGGCCTTCTTGTC
MAP0150c	CCTACGAGTATGCGCTCGAA	TCGATACGGCACTTCATGTC
MAP0216	GAAGTTCCAGGACGCCTACA	ACTGCAGGTCAGGCTTCATC
MAP3531c	TACAACGGTTGGGACATCAA	AGGTGTAGTTTTGCCCGTTG
MAP1122	CGCAGGAAATCATGACTGAG	TTAGGAGCCGAACTTTTCCA

Table S3: Putative IdeR regulated genes repressed in the presence of iron

MAP ORF ID	Predicted function/gene name	[a] Fold change (log_2)	
		cMAP	sMAP
Siderophore biosynthesis			
MAP2172c	non-ribosomal peptide synthase	3.58	1.35
MAP2173c	*mbtE*	5.35	1.28
MAP2174c	*mbtD*	3.52	1.21
MAP2175c	*mbtC*	4.65	1.26
MAP2177c	*mbtB*	5.28	1.3
MAP2205c	*mbtI*	6.32	1.95
Siderophore/metal ion transport			
MAP2413c	high affinity iron importer, *irtB*	9.52	1.42
MAP2414c	high affinity iron importer, *irtA*	18.56	1.6
MAP3778	ATPase, AAA family protein	2.73	1.98
Fattyacid metabolism			
MAP1553c	*fadE14*	3.9	1.63
MAP1554c	*fadD33_2*	3.68	2.17
MAP1555c	acyl carrier protein	3.84	2.22
Conserved hypotheticals			
MAP1594c	bacterioferritin associated ferredoxin	13.24	2.14
MAP1559c	GntR family transcriptional regulator	1.89	1.78

[a] MAP oligoarray was used to measure gene expression in the cultures of MAP strains – cattle (cMAP) or sheep (sMAP) grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of LI/HI. Genes that demonstrated the presence of 1.5 times or more of transcripts in LI compared to HI are listed. Genes are annotated based on the motif searches in KEGG database.

Table S4: IdeR regulated genes -M. smegmatis (Mc2155) microarray

	MSS ORF ID	MAP ORF ID	Predicted function/gene name	[a]Fold change (log_2)	
				cIdeR	sIdeR
Siderophore biosynthesis					
	MSMEG4509	MAP2170c	*mbtG*	-3.24	-1.21
	MSMEG4510	MAP2171c	*mbtF*	-2.55	-1.51
	MSMEG4511	MAP2173c	*mbtE*	-3.43	-1.67
	MSMEG4514	MAP2176c	thioesterase	-2.53	-1.30
	MSMEG4515	MAP2177c	*mbtB*	-3.22	-1.62
Siderophore/metal ion transport					
	MSMEG0011	MAP2414c	FAD-binding, siderophore interacting	-2.23	-1.52
	MSMEG0020	MAP3092	*fecB*, siderophore binding	-1.89	-1.65
	MSMEG0615	MAP3778	ATPase, AAA family protein	-1.87	-1.53
	MSMEG5418	MAP1762c	iron permease family protein	-2.05	-1.29
Iron storage					
	MSMEG3564	MAP1595	bacterioferritin	3.80	-1.78
	MSMEG6422	absent	bacterioferritin (bfrB)	4.93	-1.02
Transcriptional regulators					
	MSMEG0285	MAP0794	TetR family transcriptional regulator	-2.70	-2.08
	MSMEG5522	MAP1559c	GntR family transcriptional regulator	-2.82	-1.12
Conserved hypotheticals					
	MSMEG1942	MAP2073c	conserved membrane protein	2.38	1.57
	MSMEG2456	MAP0793c	possible oxidoreductase	-1.79	-1.05

MSMEG6419	MAP0192c	conserved hypothetical protein	-3.55	-1.80
MSMEG2132	MAP1555c	acyl carrier protein	-3.36	-1.62

[a]The complemented (SM3::cIdeR, SM3::sideR), mutant (SM3) and wild type (MC^2155) were grown in LB medium (**HI**) or LB medium supplemented with 2-2' dipyridyl (**LI**) (Sigma Aldrich, St. louis, MO). RNA extracted was used in microarrays as described under materials and methods. Fold change (HI/LI) for each target is calculated after normalizing the data to mutant and wildtype microarrays. A negative fold change represents repression by IdeR in the presence of iron and a positive fold change indicates de-repression of that particular target gene by IdeR in the presence of iron. c and s IdeR repressed siderophore biosynthesis and transport genes where as only cIdeR de-repressed iron storage genes in the presence of iron.

Table S5: Real time Q-PCR validation of microarray data

A

MAP ORF ID	Predicted function/gene name	[a] Fold change (log_2)	
		cMAP	sMAP
Siderophore/metal ion transport			
MAP2413c	high affinity iron importer, *irtB*	1.75	1.85
MAP2414c	high affinity iron importer, *irtA*	1.85	1.64
esx-3 secretion system			
MAP3783	esat6 family protein	1.83	1.64
MAP3784	esat6 family protein	1.77	1.76
Metabolism			
MAP1201c	acn (aconitate hydratase)	2.06	1.74
stress response regulator			
MAP0475	carD family transcriptional regulator	1.34	1.15

[a] A one step real time Q-RT PCR was used to validate microarray data of C and S MAP strains grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a log_2 ratio of **LI/HI**. Similar to microarray results most transcripts except MAP0475 are upregulated by both strains in LI. MAP0475 was upregulated only by microarray in S MAP but was not validated by Q-PCR. Genes are annotated based on the motif searches in KEGG database.

B

	MAP ORF ID	Predicted function/gene name	[a] Fold change (log_2)	
			cMAP	sMAP
Metabolism				
	MAP0150c	fadE25_2	1.83	0.52
Other poorly characterized pathways				
	MAP0216	fbpA	1.60	0.63
	MAP3531c	fbpC2	1.67	0.60
	MAP1122	integration host factor mihF	1.79	0.72

[a] A one step real time Q-RT PCR was used to validate microarray data of C and S MAP strains grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a log_2 ratio of **HI/LI**. Similar to microarray results all the transcripts were found to be upregulated only in C MAP under HI. Genes are annotated based on the motif searches in KEGG database.

Table S6: Gene expression in cattle MAP under iron-limiting conditions

	MAP ORF ID	Predicted function/gene name	[a] Fold change (log_2)
[Fe-S] cluster assembly			
	MAP1187	FeS assembly protein SufB	2.56
	MAP1188	FeS assembly protein SufD	2.73
	MAP1189	FeS assembly ATPase SufC	2.03
	MAP1190	Cysteine desulfurase	2.23
	MAP1191	Iron-sulfur assembly protein	2.53
	MAP1192	CHP	2.23
pyruvate metabolism			
	MAP2307c	pdhC	2.52
	MAP2308c	pdhB	2.36
	MAP2309c	pdhA	2.4
Lipid metabolism			
	MAP3188	fadE24	3.48
	MAP3189	fadE23	3.5
	MAP3190	fadB4	2.68
	MAP3694c	fadE5	3.15
	MAP3093	dehydrogenase	1.67
	MAP1040c	fadD9	1.74
Transporters			
	MAP3532c	ABC-type drug export system	4.05
	MAP3533c	ABC-type drug export system	3.77

esx-3 secretion system			
	MAP3780	ATPase FtsK/SpoIIIE	1.79
	MAP3781	PE family protein	1.87
	MAP3782	PPE family protein	1.74
	MAP3783	esat6 family protein	2.33
	MAP3784	esat6 family protein	2.28
	MAP3785	conserved hypothetical protein	2.3
	MAP3786	mycobacterial secretion protein	2.3
	MAP3787	membrane anchored protein	1.77
*cattle MAP specific insertion			
	MAP2325	enhanced intracellular survival protein	1.52

[a] MAP oligoarray was used to measure gene expression in the cultures of cattle MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of LI/HI. Genes that demonstrated the presence of 1.5 times or more of transcripts in LI compared to HI are listed. Genes are annotated based on the motif searches in KEGG database.

Table S7: Gene expression in sheep MAP under iron-limiting (LI) conditions

	MAP ORF ID	Predicted function/gene name	[a] Fold change (log_2)
esx-3 secretion system			
	MAP3781	PE family protein	1.56
	MAP3783	esat6 family protein	2.11
	MAP3784	esat6 family protein	2.21
	MAP3785	conserved hypothetical protein	1.94
Isoprenoid synthesis			
	MAP0476	ispD	2.12
	MAP0477	ispF	1.57
Toxin-antitoxin system			
	MAP2027c	PIN domain containing protein	2.48
	MAP2028c	PhDYeFM domain containing protein	2.09
Stress response regulators/proteins			
	MAP0475	carD family transcriptional regulator	2.21
	MAP0423	cold-shock protein (cspA1)	1.74
	MAP0669	cold-shock protein (cspA2)	2.5
	MAP3701c	heat shock protein	2.49
Molecular chaperones			
	MAP3840	dnaK	1.82
	MAP3841	grpE – functions with DnaK	2.07
	MAP3842	dnaJ	1.69
Transcription/translation			

	MAP4130	DNA-directed RNA polymerase subunit beta	1.56
	MAP4228	translation initiation factor IF-1	1.57
Lipid metabolism			
	MAP0097c	Fatty acid desaturase	1.6
	MAP2437	methylmalonyl-CoA epimerase	1.72
Redox system			
	MAP1196	trxB, thioredoxin	1.85
conserved hypotheticals			
	MAP0100	Glutamate-cysteine ligase family 2	1.55
	MAP0192c	transcriptional regulator	3.02
	MAP1544	ABC transporter related	2.19
	MAP2076c	methyltransferase	1.5
	MAP2636	hypothetical protein	2.18

[a] MAP oligoarray was used to measure gene expression in the cultures of sMAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of LI/HI. Genes that demonstrated the presence of 1.5 times or more of transcripts in LI compared to HI are listed. Genes are annotated based on the motif searches in KEGG database.

Table S8: Gene expression in cattle MAP under iron-replete (HI) conditions

	ORF ID	Predicted function/gene name	[a] Fold change (log_2)
Cellular processes			
	MAP2942c	soluble secreted antigen MPT53 precursor	1.6
Metabolism			
	MAP0150c	fadE25_2	1.88
	MAP0172	ferredoxin-dependent glutamate synthase 1	1.81
	MAP0311c	aspartate kinase	1.92
	MAP1932	ctaE (Heme-copper oxidase subunit III)	1.82
	MAP2332c	fatty acid synthase	2.28
	MAP3404	carbamoyl-phosphate synthase	2.18
	MAP3485	kasB_2	1.8
	MAP3994	acyltransferase	2.65
	MAP4203	FAD binding domain, putative	1.93
Other poorly characterized pathways			
	MAP0216	fbpA	2.16
	*MAP0860c	hypothetical protein	1.67
	MAP1084c	Flavin-utilizing monoxygenase	1.53
	MAP1122	integration host factor mihF	2
	MAP1317c	CHP	1.96
	MAP1510	secretion protein snm4	2.04
	MAP1569	modD (Fibronectin-attachment protein (FAP)	2.72

MAP1939c	Cytochrome c oxidase	2.27
MAP2516	transporter, major facilitator family protein	2.92
MAP2976c	Predicted RNA-binding protein	2.31
MAP3296c	Transcription factor WhiB	4.85
MAP3531c	fbpC2	1.52

[a] MAP oligoarray was used to measure gene expression in the cultures of C MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a $\log_2$ ratio of HI/LI. Genes that demonstrated the presence of 1.5 times or more of transcripts in HI compared to LI are listed. Genes are annotated based on the motif searches in KEGG database. *Described earlier as a part of large sequence polymorphism (LSP4) unique to *M. avium* subsp. *paratuberculosis*. Also upregulated (not shown) included 28 genes belonging to ribosomal proteins and *esx-5* operon.

Table S9: Protein expression in cattle MAP under iron-limiting conditions

	MAP ORF ID	Predicted function	[a]Fold change
Metabolism			
	MAP0150c	FadE25_2	1.49
	MAP2123	cysK cysteine synthase	1.90
	MAP3002c	phosphopantetheine adenylyltransferase	1.57
	MAP3393c	purE phosphoribosylaminoimidazole carboxylase	1.52
	MAP3413	AldB	1.87
	MAP3424c	flavoprotein disulfide reductase	1.97
	MAP3455c	isocitrate dehydrogenase	1.91
	MAP3604	Mce1_2	1.56
	MAP3680c	formate dehydrogenase	1.48
Cellular processes			
	MAP0448	FtsH	1.69
	MAP1799c	polyprenol-monophosphomannose synthase	1.77
	MAP2997c	cell division initiation protein	1.52
	MAP3402	SseA thiosulfate sulphur transferase (inorganic ion transport)	2.18
Information storage and processing			
	MAP0834c	two component regulatory protein prrA	1.52
	MAP3360c	MtrA	2.00
	MAP3389c	response regulator of a two component system	1.57
Other poorly characterized pathways			

MAP0859c	metallophosphoesterase	1.54
MAP1340c	beta-lactamase like protein	1.77
MAP1906c	cell division protein MraZ	2.65
MAP2969c	conserved hypothetical protein	1.73
MAP3358c	lipoprotein lpgB; germane - sporulation protein	1.85
MAP3845	conserved hypothetical protein	1.55

[a] iTRAQ was used to quantitate protein expression in the cultures of C MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a ratio of LI/HI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of protein expression in LI compared to HI. Genes are annotated based on the motif searches in KEGG database.

Table S10: Proteins expressed in sheep MAP under iron-limiting conditions

	MAP ORF ID	Predicted function	[a]Fold change
Metabolism			
	MAP1645	flavin-dependent oxidoreductase	1.52
	MAP1421	AsnB_1, asparagine synthase	1.54
	MAP3060c	electron transfer flavoprotein	1.55
	MAP1021c	acetyl-CoA acetyltransferase	1.56
	MAP0593c	Histidine triad family protein	1.57
	MAP0897	succinyl-CoA synthetase alpha subunit	1.59
	MAP2436c	acetyl-CoA acetyltransferase	1.63
	MAP1209	3-oxoacyl-(acyl-carrier-protein) reductase	1.70
	MAP0892c	chorismate mutase	1.74
	MAP3831c	Fe-S oxidoreductase	1.98
	MAP1159c	acyl-CoA synthase	2.30
	MAP1916c	aldolase	2.31
	MAP3443	succinate dehydrogenase flavoprotein	2.65
Cellular processes			
	MAP2794	SelR domain containing protein; oxidoreductase	1.51
	MAP0872	PhoS2_2 , ABC type transporter	1.52
	MAP1540	FHA domain containing protein	1.69
	MAP2837c	peptidoglycan biosynthesis	1.75
	MAP1434	Rieske [2Fe-2S] domain protein	1.81

	MAP4264	co-chaperonin GroES	2.15
	MAP0904	cell wall associated protein	2.23
	MAP2487c	carbonic anhydrase	1.63
Information storage and processing			
	MAP0340c	LigD domain containing protein; invovlved in DNA repair	1.55
	MAP0005	DNA topoisomerase IV subunit B	1.56
	MAP2821	endoribonuclease	1.57
	MAP3355c	ribosome-associated inhibitor A	1.76
	MAP4126	50S ribosomal protein L7/L12	1.77
	MAP3414	TetR family transcriptional regulator	1.83
	MAP2955c	elongation factor Ts	1.86
Poorly characterized pathways			
	MAP2914c	acetyltranaferase	1.51
	MAP3934c	cupin2 domain containing protein	1.53
	MAP2537	short chain dehydrogenase	1.54
	MAP2411	pyridoxine phosphate oxidase	1.54
	MAP1263	conserved hypothetical protein	1.54
	MAP1138c	lipoprotein	1.55
	MAP3835c	lipoprotein	1.61
	MAP1569	ModD; fibronectin attachment protein	1.63
	MAP0216	FbpA	1.63
	MAP3920	tuberculin related protein	1.64
	MAP1012c	patatin-like phospholipase	1.66
	MAP3666c	chromatin assembly factor	1.82
	MAP1473c	19 KDa lipoprotein	1.82

MAP2679c	conserved hypothetical protein	1.93	

[a] iTRAQ was used to quantitate protein expression in the cultures of S MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI)** medium. Fold change for each target was calculated and represented as a ratio of LI/HI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of protein expression in LI compared to HI. Genes are annotated based on the motif searches in KEGG database.

Table S11: Protein expression in cattle MAP under iron-replete conditions

	MAP ORF ID	Predicted function	[a]Fold change
Metabolism			
	MAP3694c	FadE5	2.14
	MAP3697c	succinate dehydrogenase	2.10
Cellular processes			
	MAP4264	co-chaperonin GroES	1.87
Poorly characterized			
	MAP0467c	MhuD; mycobacterial heme utilization, degrader	1.54
	MAP1754c	universal stress protein family	2.08
	MAP2622	chromosome seggregation protein	1.75
	MAP2947	CsbD family stress protein	1.64
	MAP3094c	conserved hypothetical protein	1.78
	MAP4018c	conserved hypothetical protein	2.42
	MAP4063c	nucleotide binding protein	1.50

[a] iTRAQ was used to quantitate protein expression in the cultures of cattle MAP strain grown in iron-replete (**HI**) or iron-limiting (**LI**) medium. Fold change for each target was calculated and represented as a ratio of HI/LI. Shown are the MAP genes that demonstrated the presence of 1.5 times or more of protein expression in HI compared to LI. Genes are annotated based on the motif searches in KEGG database.

Table S12: Ileum specific genes

	ORF ID	Putative function	Fold change	Regulation
Energy production and conversion				
	MAP1483c	3-ketosteroid-delta-1-dehydrogenase	2.34	down
	MAP3698c	succinate dehydrogenase flavoprotein	2.36	down
	MAP3909c	luciferase like monooxygenase	1.83	down
Amino acid transport and metabolism				
	MAP2488	ABC transporters	1.95	down
	MAP3192	alpha/beta hydrolase family protein	1.39	up
	MAP3743	putative dehydrogenase, iron-regulated protein	2.53	down
Carbohydrate transport and metabolism				
	MAP0464	phosphoglycerate mutase	1.86	down
	MAP1720	NAD-dependent epimerase	1.79	down
	MAP4076	alpha-1,2-mannosidase family protein	2.24	down
Coenzyme metabolism				
	MAP3074	methyltransferase	1.99	down
	MAP4052	menaquinone biosynthesis protein	1.77	up
Lipid metabolism				
	MAP0097c	fatty acid desaturase	2.67	down
	MAP0556c	acyl-CoA synthetase fadD17	2.07	up
	MAP1707	acyl carrier protein	2.25	down
	MAP2298c	glycerol-3-phosphate acyltransferase	2.77	down
	MAP3325	short chain dehydrogenase	3.08	down
	MAP3577	short-chain dehydrogenase/reductase SDR	3.26	up

MAP4109	(3R)-hydroxyacyl-ACP dehydratase	2.24	down
Translation, ribosomal structure and biogenesis			
MAP2470c	arginyl-tRNA synthetase	2.23	down
MAP3767c	30S ribosomal protein S18	2.50	down
Transcription			
MAP1736	TetR family transcriptional regulator	2.82	down
MAP2418	Glucitol operon activator protein	1.52	down
MAP2591	TetR family transcriptional regulator	3.58	down
MAP2836	LexA repressor	2.49	up
MAP2963c	XRE family transcriptional regulator	3.06	down
MAP3967	XRE family transcriptional regulator	2.87	up
DNA replication, recombination and repair			
MAP2493c	hydrolase	1.63	up
Cell envelope biogenesis, outer membrane			
MAP0586c	Lytic Transglycosylase	1.81	up
MAP3245	putative glycosyl transferase	4.97	up
Not in COGs			
MAP0075	Mycobacterium membrane protein	2.72	down
MAP0090c	twin-arginine translocation pathway signal protein	2.42	down
MAP0114	heat shock protein	3.46	down
MAP0210c	exported repetitive protein precursor Erp	6.19	up
MAP0852	Sugar transport protein	3.78	down
MAP0864	FAD linked oxidase domain protein	3.40	down
MAP1379	putative 6-phosphofructokinase	2.76	down

	MAP1833c	putative DNA-binding protein	2.25	down
	MAP2152c	putative TonB-dependent receptor protein	3.66	down
	MAP2522	putative lipoprotein	2.76	down
	MAP2624c	mannosyltransferase	2.47	up
	MAP2769c	4'-phosphopantetheinyl transferase	5.93	down
	MAP2843c	integral membrane protein	1.61	up
	MAP3753	amino acid adenylation domain-containing protein	5.99	down
	MAP3942	putative chorismate synthase	2.12	up
	MAP4034	MreB family protein	3.42	up
	MAP4120c	FAD dependent oxidoreductase	1.97	up
Inorganic ion transport and metabolism				
	MAP1484c	Rieske (2Fe-2S) domain protein	2.11	down
	MAP1922c	anion-transporting ATPase	2.00	up
Secondary metabolites biosynthesis, transport and catabolism				
	MAP0547	cytochrome P450	2.45	down
	MAP2173c	peptide synthetase mbtE	3.24	up
General function prediction only				
	MAP0622c	Metallo-beta-lactamase	1.71	up
	MAP0743c	amidohydrolase	1.68	down
	MAP4296c	putative transcriptional regulator	1.86	up
Function unknown				
	MAP0437	Aminoacyl-transfer RNA synthetases	1.74	up
	MAP0610c	RelA/SpoT domain-containing protein	3.44	up
	MAP2024c	carboxymuconolactone decarboxylase	2.31	down

Figure S13: Mesenteric lymphnode specific genes

	ORF ID	Putative function	Fold change	Regulation
Energy production and conversion				
	MAP1171	NADPH oxidoreductase	1.42	down
	MAP2620c	nitrate reductase	1.90	down
	MAP3032c	Isocitrate/isopropylmalate dehydrogenase	2.08	down
Translation, ribosomal structure and biogenesis				
	MAP4141	30S ribosomal protein S7	2.17	down
Transcription				
	MAP1634	LysR transcriptional regulator	4.92	down
DNA replication, recombination and repair				
	MAP0071	DnaB helicase-like protein	2.09	down
Not in COGs				
	MAP0033c	transcription factor whiB	2.57	down
	MAP0041	integral membrane protein	4.41	down
	MAP0406	putative protease	1.52	down
	MAP0736	putative transcription regulator	2.56	down
	MAP1788	TetR family transcriptional regulator	2.81	down
	MAP2759	amidohydrolase	3.01	down
	MAP3014	polyketide synthase	1.84	down
	MAP3342	transferrin like protein	2.47	down
Inorganic ion transport and metabolism				
	MAP0144	ABC transporter protein	2.88	down
	MAP0982c	arsenate reductase	2.56	down

	MAP3141c	2Fe2S domain containing protein	2.22	down
General function prediction only				
	MAP0722	amidohydrolase	2.62	down
	MAP2831	proteasome protease	1.77	down
Function unknown				
-	MAP0796c	putative monooxygenase	2.62	down

Figure S14: Ileum and Mesenteric lymphnode shared genes

	ORF ID	Putative function	Fold change	Regulation
Energy production and conversion				
	MAP1491	L-carnitine dehydratase	1.89	down
	MAP1934	Fe-S containing protein	2.95	down
	MAP2451c	F0F1 ATP synthase subunit beta	1.77	down
	MAP3545	oxidoreductase FAD-binding region	2.83	down
	MAP3956	dihydrolipoamide dehydrogenase	3.67	down
Cell division and chromosome partitioning				
	MAP3172c	cell division transport system ATP-binding protein	3.24	down
Amino acid transport and metabolism				
	MAP0302c	pyridoxal-phosphate-dependent transferase	3.38	up
	MAP3898	putative thiamine biosynthesis oxidoreductase	1.87	up
Nucleotide transport and metabolism				
	MAP2420c	putative dNTP pyrophosphatase	1.83	up
	MAP3820	deoxycytidine triphosphate deaminase	4.50	down
Carbohydrate transport and metabolism				
	MAP1270c	alpha amylase	2.74	down
Coenzyme metabolism				
	MAP1816c	precorrin-4 C11-methyltransferase	2.69	down
Lipid metabolism				
	MAP1018c	enoyl-CoA hydratase	3.32	down
	MAP1485c	Acyl-protein synthetase, LuxE	2.03	down
	MAP1715	fatty oxidation protein FadB	2.53	down

	MAP2041	alpha beta hydrolase	2.39	down
	MAP2312c	acyl-CoA dehydrogenase FadE19	3.63	down
	MAP3137c	short chain dehydrogenase	2.21	down
	MAP4109	(3R)-hydroxyacyl-ACP dehydratase subunit HadC	2.24	down
Translation, ribosomal structure and biogenesis				
	MAP0459	lysyl-tRNA synthetase	2.48	down
	MAP1246	isoleucyl-tRNA synthetase	6.14	up
	MAP3769c	50S ribosomal protein L33	2.36	down
	MAP4160	30S ribosomal protein S10	2.00	down
	MAP4161	50S ribosomal protein L3	2.00	down
	MAP4179	50S ribosomal protein L5	1.70	down
Transcription				
	MAP1446c	IclR family transcriptional regulator	3.01	up
	MAP1832c	putative transcriptional regulator	2.44	up
	MAP2442	LysR transcriptional regulator	4.79	up
	MAP3077	MarR family transcriptional regulator	1.66	down
	MAP3139c	MarR family transcriptional regulator	2.22	down
	MAP3323c	anti sigma factor	2.85	down
DNA replication, recombination and repair				
	MAP0866	DNA breaking-rejoining enzyme	4.23	down
	MAP2521c	ATP-dependent RNA helicase DeaD	2.96	down
	MAP2768c	phage integrase family protein	6.39	down
Cell envelope biogenesis, outer membrane				
	MAP1905c	methyltransferase	8.13	up
	MAP3019c	D-alanyl-alanine synthetase A	2.33	up
	MAP3979	glycosyl transferase	2.70	up

Not in COGs

MAP0099	CsbD like stress protein	3.74	down
MAP0102	molybdenum cofactor biosynthesis	3.32	down
MAP0233c	Arabinofuranosyltransferase	2.04	up
MAP0282c	cell divisionFtsK/SpoIIIE	3.03	down
MAP0343	Bmp family lipoprotein	1.72	down
MAP0443c	HNH endonuclease	2.54	up
MAP0658c	fatty acid desaturase DesA1	2.45	down
MAP0768c	cell division protein	3.88	down
MAP0914c	polyketide synthase	1.76	up
MAP1170	fattyacid desaturase	1.34	down
MAP1218c	cell division protein/spoIIIE	3.36	down
MAP1233	FkbM family methyltransferase	3.05	down
MAP1343	phosphofructokinase	2.31	down
MAP1517	ABC type transport protein	2.90	down
MAP1522	PPE family protein	2.22	up
MAP1794	probable transcriptional regulator	2.25	down
MAP1857	heat shcok protein	2.57	down
MAP2202c	fattyacid desaturase	3.21	down
MAP2498c	putative lipoprotein LprB	2.55	down
MAP2543	predicted transcriptional regulator	2.88	down
MAP2678c	TetR family transcriptional regulator	3.49	down
MAP2679c	hemerythrin HHE cation binding domain protein	3.20	down
MAP2758	AAA ATPase	2.64	down
MAP2763c	serine/threonine-protein kinase	2.28	down
MAP3345c	sigma 70	2.41	down

	MAP3364c	alkane-hydroxylase	2.54	up
	MAP3741	non-ribosomal peptide synthase	2.67	down
	MAP3813	RNA polymerase	1.88	down
	MAP4326c	putative phage like protein	3.87	down
	MAP4328c	serine/threonine-protein kinase	3.30	down
Inorganic ion transport and metabolism				
	MAP1864c	siderophore interacting protein	2.00	down
	MAP3731c	ABC type transport protein	6.01	down
	MAP3773c	Ferric uptake regulator, FuR	3.88	down
Secondary metabolites biosynthesis, transport and catabolism				
	MAP0109	virulence factor mce family mce1B	2.07	down
	MAP0565	virulence factor mce family	2.57	down
	MAP0567	virulence factor mce family	3.08	up
	MAP0752c	cytochrome P450	2.08	down
	MAP0758	ABC type transport protein	3.54	down
	MAP1851	virulence factor mce family protein	2.59	down
	MAP1869c	thioesterase - role in non ribosomal peptide synthesis	3.79	down
	MAP2117c	integral membrane protein	3.32	down
	MAP2192	virulence factor mce family protein	2.63	down
General function prediction only				
	MAP0076	putative transport protein MmpL4	2.96	down
	MAP1321c	putative transcriptional regulator	1.79	up
	MAP1654c	2-nitropropane dioxygenase	12.61	up
	MAP1738	transport protein mmpL5	3.91	down
	MAP2319c	ATP binding protein	2.39	down

	MAP3035	NADPH quinone oxidoreductase	1.97	down
Function unknown				
	MAP0434	integral membrane protein TerC family	2.58	up
	MAP1006	predicted helicase	3.93	down
	MAP1845c	integral membrane protein	1.53	down
	MAP2222c	AAA ATPase	1.69	down
	MAP2523c	homoserine dehydrogenase	3.19	down
	MAP2740	Iron permease FTR1 family protein	3.13	up
	MAP3812c	putative lipoprotein	3.97	down
	MAP4269c	Phage derived protein Gp49-like	2.08	down
Signal transduction mechanisms				
	MAP3407c	anti sigma factor	1.72	down
Defense mechanisms				
	MAP3162c	beta-lactamase	2.18	down

Table S15: Macrophage specific genes (6 and 48hrs PI)

	ORF ID	Putative function	Fold change	Regulation
Cell division and chromosome partitioning				
	MAP2990c	Chromosome segregation protein SMC	3.04	up
Amino acid transport and metabolism				
	MAP0533	4-hydroxy-2-ketovalerate aldolase	4.26	up
Coenzyme metabolism				
	MAP2036	phosphoadenosine phosphosulfate reductase	4.41	down
Cell motility and secretion				
	MAP1515	PPE family protein	6.25	down
Not in COGs				
	MAP1606c	cyclase/dehydrase superfamily protein	2.04	up
	MAP2379	Thioesterase superfamily	7.05	down
	MAP2795	predicted transcriptional regulator	6.05	down
	MAP3477	acetyl transferase	3.04	down
Posttranslational modification, protein turnover, chaperones				
	MAP3554c	methionine sulfoxide reductase A	2.30	up
Inorganic ion transport and metabolism				
	MAP3776c	ferric siderophore transporter	5.76	down
	MAP4171	Arylsulfatase A and related enzymes (*ats*A)	3.94	up
Secondary metabolites biosynthesis, transport and catabolism				
	MAP1614c	cytochrome P450	3.53	down

Table S16: Macrophage specific genes (6 and 120hrs PI)

	ORF ID	Putative function	Fold change	Regulation
Amino acid transport and metabolism				
	MAP3873	O-succinylhomoserine sulfhydrylase	1.99	up
Carbohydrate transport and metabolism				
	MAP2434	glycogen branching enzyme	6.98	up
Coenzyme metabolism				
	MAP2036	phosphoadenosine phosphosulfate reductase	4.41	down
Lipid metabolism				
	MAP2101	acyl-CoA dehydrogenase-like protein	3.40	up
	MAP2896c	Acyl-CoA dehydrogenases (*fad*E21)	3.75	up
Translation, ribosomal structure and biogenesis				
	MAP0067	30S ribosomal protein	2.59	up
	MAP1063	aspartyl-tRNA synthetase	4.06	up
Transcription				
	MAP4312	TetR family transcriptional regulator	2.54	down
Cell motility and secretion				
	MAP1515	PPE family proteins	6.25	down
Posttranslational modification, protein turnover, chaperones				
	MAP0167	ATPase central domain-containing protein	4.92	up
Inorganic ion transport and metabolism				
	MAP4171	Arylsulfatase A and related enzymes	3.94	up
Function				

unknown				
	MAP4063c	nucleotide-binding protein	2.52	up
Signal transduction mechanisms				
-	MAP3472c	putative regulatory protein	1.64	down

Table S17: Macrophage specific genes (48 and 120hrs PI)

	ORF ID	Putative function	Fold change	Regulation
Energy production and conversion				
	MAP1168c	putative oxidoreductase	5.09	down
Amino acid transport and metabolism				
	MAP0277c	prephenate dehydrogenase	2.17	down
	MAP1859c	B12-dependent methionine synthase	2.29	up
	MAP3025c	isopropylmalate isomerase small subunit	2.55	down
	MAP3684	oxidoreductase	2.40	down
Nucleotide transport and metabolism				
	MAP0639	phosphoribosylaminoimidazole synthetase	2.12	down
Carbohydrate transport and metabolism				
	MAP4121	alpha-mannosidase	2.55	up
Coenzyme metabolism				
	MAP2036	phosphoadenosine phosphosulfate reductase	1.73	down
Lipid metabolism				
	MAP0912c	acetyl-CoA carboxylase	1.53	down
	MAP2313c	Acetyl/propionyl-CoA carboxylase	1.96	up
Translation, ribosomal structure and biogenesis				
	MAP0459	lysyl-tRNA synthetase	4.41	up
	MAP2682c	translation-associated GTPase	2.29	up
	MAP3453c	tryptophanyl-tRNA synthetase	4.81	up
	MAP4235	tRNA pseudouridine synthase A	1.65	down
Transcription				

MAP0475	CarD family transcriptional regulator	4.47	down
DNA replication, recombination and repair			
MAP1257	DNA polymerase III subunit alpha	2.68	up
Cell envelope biogenesis, outer membrane			
MAP1203	Cell wall-associated hydrolases	3.62	up
Cell motility and secretion			
MAP1515	PPE family protein	4.61	down
Not in COGs			
MAP0140	PE family protein	2.76	up
MAP0185c	Alpha-amylase signature	4.05	up
MAP0640c	conserved hypothetical protein	3.81	down
MAP2835c	LysM domain containing protein	2.66	down
MAP3259	transmembrane serine/threonine-protein kinase J	2.68	up
MAP3626c	NF-X1 finger transcription factor	3.20	down
Inorganic ion transport and metabolism			
MAP2046	thiosulfate sulfurtransferase	1.92	down
MAP4171	Arylsulfatase A and related enzymes	3.34	up
Secondary metabolites biosynthesis, transport and catabolism			
MAP0220	polyketide synthase	2.16	down
MAP0830c	FAD dependent oxidoreductase	2.26	down
General function prediction only			
MAP0136	glyoxalase family protein	1.74	down
MAP0372	PfpI family intracellular peptidase	1.89	up
MAP0404c	hydrolase ephE	5.42	down

	MAP0541c	ketosteroid isomerase- like protein	1.61	up
Function unknown				
	MAP1704c	glyoxylase family protein	1.90	down
	MAP2119	ABC transporter, permease protein OppB	2.89	up

Table S18: Common genes between natural and in vitro infection

		Macrophages		Tissues	
ORF ID	Putative function	Fold change	Regulation	Fold change	Regulation
Energy production and conversion					
MAP0367	molybdopterin oxidoreductase	3.02	up	2.27	down
MAP0369	4Fe-4S ferredoxin, iron-sulfur binding protein	3.67	up	2.02	up
MAP1504	ferredoxin	2.65	up	2.36	down
MAP1712	L-carnitine dehydratase	2.73	up	2.70	down
MAP2307c	dihydrolipoamide S-acetyltransferase E2 component PdhC	2.37	up	2.39	down
MAP3120c	luciferase-like protein	3.23	down	2.26	up
MAP4071	galactose-1-phosphate uridylyltransferase	3.31	down	2.93	up
Aminoacid transport and metabolism					
MAP1727	alpha/beta fold family hydrolase	2.01	up	6.04	down
MAP2294c	NAD-glutamate dehydrogenase	2.20	down	2.03	down
MAP2485c	sulfate adenylyltransferase	2.35	down	2.00	down
MAP3025c	isopropylmalate isomerase small subunit	2.03	up	2.27	down
MAP3037c	acetolactate synthase	3.03	down	2.45	down
MAP3997c	SerB family protein	2.54	up	2.01	down
Nucleotide transport and metabolism					
MAP0279	cytidine/deoxycytidylate deaminase	2.50	down	3.85	up
Carbohydrate transport and metabolism					
MAP0876c	kinase, PfkB family protein	2.51	up	2.16	up
MAP2285c	ribose-5-phosphate isomerase B	2.05	down	2.17	up
Coenzyme metabolism					

	MAP1274	adenosylmethionine transaminase	2.14	down	2.57	up
	MAP1291	nicotinate-nucleotide pyrophosphorylase	2.57	down	3.14	up
	MAP1873c	acetolactate synthase	5.26	up	3.50	down
	MAP3760c	putative methyltransferase	3.38	up	2.76	down
Lipid metabolism						
	MAP0097c	fatty acid desaturase	6.43	up	2.67	down
	MAP0150c	acyl-CoA dehydrogenase FadE25_2	2.88	down	9.48	up
	MAP0543c	lipid transfer protein	3.60	up	2.25	down
	MAP1715	fatty oxidation protein FadB	3.98	up	2.53	down
	MAP2101	acyl-CoA dehydrogenase	2.76	down	3.42	down
	MAP2398	enoyl-CoA hydratase/isomerase family protein	2.80	down	2.43	up
	MAP2530	short-chain dehydrogenase/reductase SDR	3.14	up	4.13	down
	MAP3087c	enoyl-CoA hydratase	2.07	up	3.41	up
	MAP3960	acyl-ACP thioesterase	2.30	down	2.13	down
	MAP3996c	MaoC-like dehydratase	3.25	down	3.18	down
	MAP4107	(3R)-hydroxyacyl-ACP dehydratase subunit HadA	4.29	up	2.77	down
Translation, ribosomal structure and biogenesis						
	MAP0459	lysyl-tRNA synthetase	3.27	down	2.48	down
	MAP1481c	ATP-dependent RNA helicase	3.01	down	4.56	up
	MAP3453c	transferrin like protein	4.18	down	2.31	down
Transcription						
	MAP0375c	stage II sporulation protein E (SpoIIE)	2.28	down	3.53	up
	MAP0661c	ArsR family transcriptional regulator	2.30	down	2.98	down
	MAP1027c	transcription elongation factor	4.96	up	2.06	down

	MAP1631c	TetR family transcriptional regulator	3.05	up	2.80	down
	MAP1634	LysR family transcriptional regulator	2.31	up	4.92	down
	MAP1726c	TetR family transcriptional regulator	2.19	up	2.64	down
	MAP1736	TetR family transcriptional regulator	3.20	down	2.82	down
	MAP1814	RNA polymerase sigma factor SigC	2.10	up	2.53	up
	MAP1832c	proteasome accessory factor B	2.62	up	2.44	up
	MAP2963c	XRE family transcriptional regulator	3.02	up	3.48	down
	MAP3843	MerR family transcriptional regulator	2.29	down	7.14	up
	MAP3967	XRE family transcriptional regulator	3.94	down	2.87	up
DNA replication, recombination and repair						
	MAP0005	DNA gyrase subunit B	2.11	down	2.86	down
	MAP0866	integrase family protein	3.45	up	4.23	down
	MAP1257	DNA polymerase III subunit alpha	2.23	up	2.00	down
	MAP2964c	integrase/recombinase	2.04	up	2.44	down
Cell envelope biogenesis, outer membrane						
	MAP1231	GDP-mannose 4,6-dehydratase	4.12	up	3.32	down
	MAP3963	mycolic acid synthase UmaA	2.27	down	3.04	down
Not in COGs						
	MAP0102	molybdenum cofactor biosynthesis	6.30	up	3.32	down
	MAP0233c	putative arabinofuranosyltransferase	2.35	up	2.04	up
	MAP0555c	RTX toxins and related Ca2+-binding protein	4.79	down	2.06	down
	MAP0616c	DoxX family protein	8.70	up	2.94	up
	MAP0617	TetR family transcriptional regulator	3.46	up	2.45	up
	MAP0726	HlyC/CorC family transporters	2.45	up	2.93	up
	MAP0767c	twin-arginine translocation pathway signal	3.11	up	2.03	down

MAP0851	SAM-dependent methyltransferase	2.16	up	2.40	down
MAP0856c	phosphoribosylformylglycinamidine synthase	2.90	up	2.36	down
MAP0862	DNA helicase	2.61	up	2.63	down
MAP0970	lipocalin like protein	5.18	up	2.57	down
MAP1633c	integral membrane protein	4.09	up	5.18	down
MAP1636c	putative transcriptional regulator	5.40	up	3.80	down
MAP1822c	collagen like protein	3.49	up	5.85	down
MAP2140c	calcium/proton exchanger	2.81	up	3.18	down
MAP2153	restriction endonuclease	2.72	up	2.21	down
MAP2417c	Possible lipoprotein LppJ	2.55	up	2.88	down
MAP2531	aromatic-ring hydroxylating dioxygenase	2.43	up	3.30	down
MAP2679c	FeoA family protein	2.17	up	3.20	down
MAP2758	AAA ATPase	3.13	up	2.64	down
MAP2759	Amidohydrolase family	2.53	up	3.01	down
MAP2769c	4'-phosphopantetheinyl transferase	3.63	up	5.93	down
MAP3039c	transmembrane protein mmpL3	4.47	up	2.40	up
MAP3094c	glycosyl transferase family protein	2.42	up	2.26	down
MAP3128	periplasmic ligand binding sensory protein	2.45	up	2.49	down
MAP3342	conserved hypothetical protein	3.52	down	2.47	down
MAP3436c	iron utilization protein	2.78	up	2.42	down
MAP3487c	DNA polymerase involved in UV protection	2.33	down	2.93	up
MAP3681	XRE family transcriptional regulator	3.86	up	3.18	down
MAP3761c	Cytochrome C biogenesis protein	2.95	up	2.37	down
MAP3763c	polyketide synthase associated protein PapA3	4.25	up	7.57	down
MAP3815	autophagy associated protein	6.02	up	6.35	down

	MAP3816	phage integrase family protein	2.26	up	3.49	down
	MAP4175	transmembrane protein	3.02	up	2.04	up
Posttranslational modification, protein turnover, chaperones						
	MAP0855	DNA repair protein RadA	3.56	up	2.32	down
	MAP2012c	Molecular chaperone	2.04	down	3.07	up
	MAP3778	AAA ATPase	2.90	down	2.08	down
Inorganic ion transport and metabolism						
	MAP0645c	putative thiosulfate sulfurtransferase	2.63	down	2.19	down
	MAP1110	ABC transporter ATP-binding protein	3.62	down	2.28	up
	MAP1301	calcium/proton exchanger	2.36	down	2.23	up
	MAP1922c	anion-transporting ATPase superfamily protein	2.44	down	2.00	up
	MAP2104	formate dehydrogenase H FdhF	2.31	down	2.51	down
	MAP2139	Ferric uptake regulator, FurB	2.04	up	2.42	down
	MAP3682	catalase katE	2.10	up	2.99	down
	MAP3773c	ferric uptake regulator, Fur family	2.33	up	3.88	down
	MAP4171	arylsulfatase	2.85	down	2.14	down
Secondary metabolites biosynthesis, transport and catabolism						
	MAP0566	MCE-family protein Mce4C	3.33	down	2.60	up
	MAP0598c	cytochrome P450	4.24	down	2.32	down
	MAP0760	virulence factor Mce family protein	3.43	up	2.01	down
	MAP1964c	thioesterase superfamily protein	3.48	up	2.04	down
	MAP2116c	MCE-family protein mce3A	2.93	up	3.23	down
	MAP2189	virulence factor MCE-like protein	8.48	up	2.96	down
	MAP3744	thiazolinyl imide reductase	3.27	up	2.48	down
	MAP3764c	polyketide synthase Pks2	6.85	up	4.37	down

	MAP3818	cytochrome P450	4.02	up	3.50	down
	MAP4086	mce-family protein mce2c	2.10	up	2.31	down
General function prediction only						
	MAP0076	putative transport protein MmpL4	4.20	up	3.62	down
	MAP0859c	metallophosphoesterase	2.32	up	4.78	down
	MAP0907	2,5-didehydrogluconate reductase	4.96	up	5.95	down
	MAP1707	short chain dehydrogenase	3.35	up	2.25	down
	MAP1728c	haloacid dehalogenase, type II	2.50	up	7.69	down
	MAP2232	Putative membrane protein, MmpL family	2.11	up	2.58	down
	MAP2528	short-chain dehydrogenase/reductase SDR	2.23	up	4.25	down
	MAP2718c	N-alpha-acyl-glutamine aminoacylase	2.10	up	2.04	up
	MAP2790c	ATP/GTP-binding integral membrane protein	3.50	down	2.95	up
	MAP3426c	amidohydrolase	2.07	down	2.03	up
	MAP3747c	cobalamin synthesis CobW-like protein	7.64	up	3.67	down
	MAP3751	MmpL family transport protein	2.39	up	2.19	down
	MAP3772c	cobalamin synthesis protein, P47K	2.21	up	2.31	down
Function unknown						
	MAP2196	dihydrodipicolinate reductase	5.62	up	3.13	down
	MAP2529	Carboxymuconolactone decarboxylase	3.19	up	2.54	down
	MAP3291c	transmembrane protein	2.20	down	2.93	down
Signal transduction mechanisms						
	MAP2219c	transcriptional regulator	2.79	down	2.48	down
Defense mechanisms						
	MAP3162c	beta-lactamase	2.62	up	2.18	down

Table S19: Expression of MAP lineage specific LSPs in the tissues of naturally infected cattle

LSP number[a]				
LSP4				
	ORF ID	Putative function	Fold change	Regulation
	MAP0852	putative transport protein	3.78	down
	MAP0854	replicase	2.08	down
	MAP0857c	putative beta-lactamase	4.31	down
	MAP0858	proteophosphoglycan ppg4	8.06	down
	MAP0860c	plasmid replication region DNA binding	3.79	down
	MAP0861	cytochrome c oxidase, subunit III	12.52	down
	MAP0863	putative transport protein	3.92	down
	MAP0864	acetyltransferase	3.40	down
LSP11				
	MAP2149c	ErfK/YbiS/YcfS/YnhG family protein	2.52	down
	MAP2151	calpain-like cysteine peptidase	2.38	down
	MAP2152c	molybdopterin biosynthesis enzyme	3.66	down
	MAP2154c	cupin 4 family protein	6.23	down
LSP12				
	MAP2179	lipoprotein	2.37	down
	MAP2181c	putative transcriptional regulator	2.21	down
	MAP2182c	protein with domain of unknown function	3.35	down
	MAP2183c	cytochrome p450	2.82	down

MAP2190	virulence factor MCE-like protein	4.22	down
MAP2192	virulence factor MCE-like protein	2.63	down
MAP2193	virulence factor MCE-like protein	2.29	down
MAP2194	virulence factor MCE-like protein	3.14	down
MAP2195	hypothetical protein	2.87	down

LSP14

MAP3730	methyltransferase	2.12	down
MAP3731c	ABC type transporter	6.01	down
MAP3732c	trasporter protein	6.36	down
MAP3733c	integral membrane protein	5.07	down
MAP3734c	ABC type multidrug transporter protein	4.11	down
MAP3735c	putative ferric siderophore transporter	5.04	down
MAP3736c	ABC type transporter	3.46	down
MAP3737	PPE family protein	2.09	down
MAP3738c	methyltransferase	3.78	down
MAP3741	non-ribosomal peptide synthase	2.67	down
MAP3742	non-ribosomal peptide synthase	3.85	down
MAP3743	putative dehydrogenase, iron-regulated protein	2.53	down
MAP3745	thioesterase	5.05	down
MAP3746	protein with uncharacterized domain	2.73	down
MAP3749	short chain dehydrogenase	5.95	down
MAP3751	mmpL family protein mmpL4_5	2.19	down
MAP3752	acyl coA synthase fadD28	2.91	down

	MAP3753	non-ribosomal peptide synthase	5.99	down
	MAP3756c	oxidoreductase	3.78	down
	MAP3757c	alpha beta hydrolase	2.41	down
LSP15				
	MAP3771	50S ribosomal protein L31 type B	4.23	down
	MAP3774c	ABC type ferric siderophore transporter	7.01	down
	MAP3775c	ABC type metal transporter	5.08	down
LSP16				
	MAP3814c	transposase IS116/IS110/IS902 family	5.15	down
	MAP3817c	putative membrane protein	2.56	down
Deletion 1				
	MAP1484c	Rieske (2Fe-2S) domain-containing protein	2.11	down
	MAP1485c	acyl-CoA synthase	2.03	down
	MAP1490	CoA-transferase family III	2.06	down
Deletion 2				
	MAP1730c	cobalamin synthesis protein	3.55	down
	MAP1734	PPE family protein	2.98	up
	MAP1737	mycobacterium membrane protein mmps5	5.22	down
	MAP1738	mmpL family protein mmpL5	3.91	down

Table S20: Expression of MAP lineage specific LSPs in the in vitro infected macrophages

LSP number[a]				
LSP4				
	ORF ID	Putative function	Fold change	Regulation
	MAP0851	putative ribonuclease	2.16	up
	MAP0853	aldehyde dehydrogenase	5.49	up
	MAP0855	putative transport protein	9.64	up
	MAP0856c	phosphoribosylformylglycinamidine synthase	5.47	up
	MAP0859c	metallophosphatase	4.70	up
	MAP0862	acyl co-A transferase	4.12	up
	MAP0865	FtsK; DNA segregation ATPase	11.26	up
	MAP0866	DNA breaking-rejoining enzyme	6.54	up
LSP11				
	MAP2153	restriction endonuclease	2.72	up
LSP12				
	MAP2176c	Thioesterase and alpha beta hydrolase	3.97	up
	MAP2180c	beta-lactamase	4.39	up
	MAP2185c	amidohydrolase	10.99	up
	MAP2189	virulence factor MCE-like protein	8.48	up
	MAP2196	dihydrodipicolinate reductase	5.62	up
LSP14				

	MAP3729	Taurine catabolism	2.85	up
	MAP3739c	major factilitator superfamily transport protein	6.06	up
	MAP3744	oxidoreductase family protein	7.42	up
	MAP3747c	cobalamin synthesis protein	7.64	up
	MAP3751	MMPL family transport protein	2.39	up
	MAP3760c	methyl transferase	3.38	up
	MAP3761c	cytochrome biogenesis protein	20.15	up
	MAP3762c	glycosyl transferase	3.16	up
	MAP3763c	polyketide synthase associated protein	9.21	up
	MAP3764c	non-ribosomal peptide synthase	6.85	up
LSP15				
	MAP3772c	cobalamin synthesis protein	3.78	up
	MAP3773c	Ferric Uptake Regulator	5.18	up
	MAP3776c	ABC type metal transporter	6.45	up
LSP16				
	MAP3815	exinuclease	12.91	up
	MAP3816	DNA breaking-rejoining enzyme	9.02	up
	MAP3818	cytochrome p450	4.64	up
Deletion 2				
	MAP1728c	yfnB, predicted hydrolase	10.75	up
	MAP1731c	Fumarylacetoacetate (FAA) hydrolase family	3.93	up
	MAP1735	alpha beta hydrolase	4.82	up

MAP1736	transcriptional regulator	3.20	down
MAP1743c	nitroreductase	6.35	up

Figure S1: Multiple Sequence alignment of MAP2827c, MAP2827, MAV3604 and RV2711

```
Sheep MAP2827  MNDLVDTTEMYLRTIYDLEEEGVTPLRARIAERLDQSGPTVSQTVSRMERDGLLHVAGDR  60
MAV3604        MNDLVDTTEMYLRTIYDLEEEGVTPLRARIAERLDQSGPTVSQTVSRMERDGLLHVAGDR  60
Cattle MAP2827 MNDLVDTTEMYLRTIYDLEEEGVTPLRARIAERLDQSGPTVSQTVSRMERDGLLHVAGDR  60
RV2711         MNELVDTTEMYLRTIYDLEEEGVTPLRARIAERLDQSGPTVSQTVSRMERDGLLRVAGDR  60
MAP2827c       ----------------------MTAWAISCGRVTFSWPGMTIVTP----------AGP- 26

Sheep MAP2827  HLELTDKGRALAVAVMRKHRLAERLLVDVIRLPWEEVHAEACRWEHVMSEDVERRLVKVL 120
MAV3604        HLELTDKGRALAVAVMRKHRLAERLLVDVIRLPWEEVHAEACRWEHVMSEDVERRLVKVL 120
Cattle MAP2827 HLELTDKGRALAVAVMRKHRLAERLLVDVIGLPWEEVHAEACRWEHVMSEDVERRLVKVL 120
RV2711         HLELTEKGRALAIAVMRKHRLAERLLVDVIGLPWEEVHAEACRWEHVMSEDVERRLVKVL  120
MAP2827c       ---VSTVTRALGTTPASLSREIRSMSPWTCSVSWRTTTATG---------APDGNSVSRT 74

Sheep MAP2827  NNPTTSPFGNPIPGLLDLGVGPESGAEEANLVRLTELPSGAPVAVVVR---QLTEHVQGD  177
MAV3604        NNPTTSPFGNPIPGLLDLGVGPESGAEEANLVRLTELPSGAPVAVVVR---QLTEHVQGD  177
Cattle MAP2827 NNPTTSPFGNPIPGLLDLGVGPESGAEEANLVRLTELPSGAPVAVVVR---QLTEHVQGD  177
RV2711         NNPTTSPFGNPIPGLVELGVGPEPGADDANLVRLTELPAGSPVAVVVR---QLTEHVQGD  177
MAP2827c       RLASSAPDSGPTPRSSSPGIGLPNGEVVGLFSTLTRRRSTSSLITCSQRHASACTSSQGS  134

Sheep MAP2827  IDLISRLKDAGVVPNARVTVETGPAG-VTIVIPGHENVTLPHEMAHAVKVEKV--- 229
MAV3604        IDLISRLKDAGVVPNARVTVETGPAG-VTIVIPGHENVTLPHEMAHAVKVEKV--- 229
```

```
Cattle MAP2827  IDLISRLKDAGVVPNARVTVETGPAG-VTIVIPGHENVTLPHEMAHAVKVEKV---  229
RV2711          IDLITRLKDAGVVPNARVTVETTPGGGVTIVIPGHENVTLPHEMAHAVKVEKV---  230
MAP2827c        RMTSTSSRSASRCLRITATASARPLS-VSSRWRSPATCSSPSRSIRDTVWLTVGPL  189
```

Figure S2: Overall Strategy

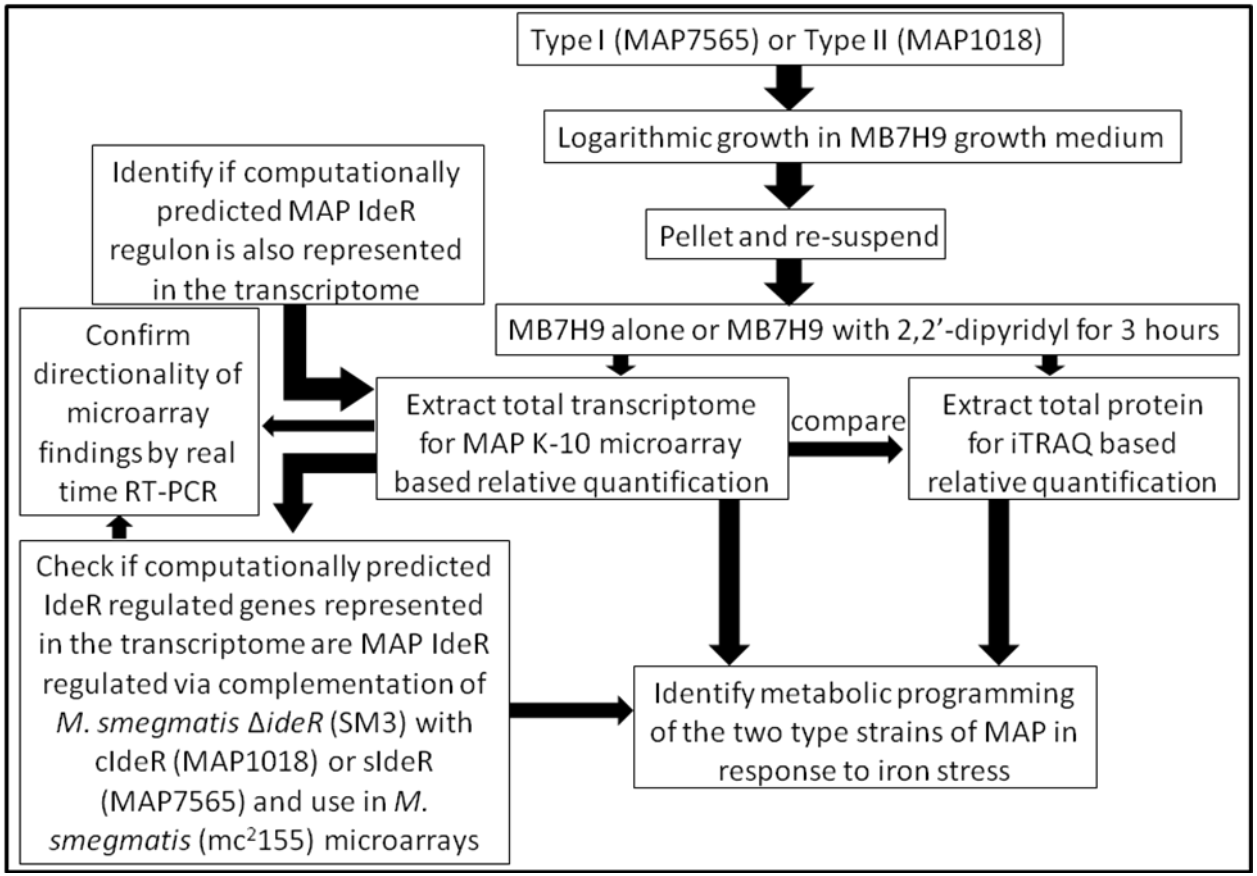

Figure S2: Shown here is the overall strategy used to profile iron dependent responses of the two type strains of MAP. MAP K-10 microarrays and iTRAQ experiments were performed as described in methods section. *M. smegmatis* arrays were used to confirm previously predicted MAP IdeR regulated genes and also to identify differences between the two MAP IdeRs.

Figure S3: iTRAQ experiment design for proteome profiling of the two type strains of MAP in response to iron

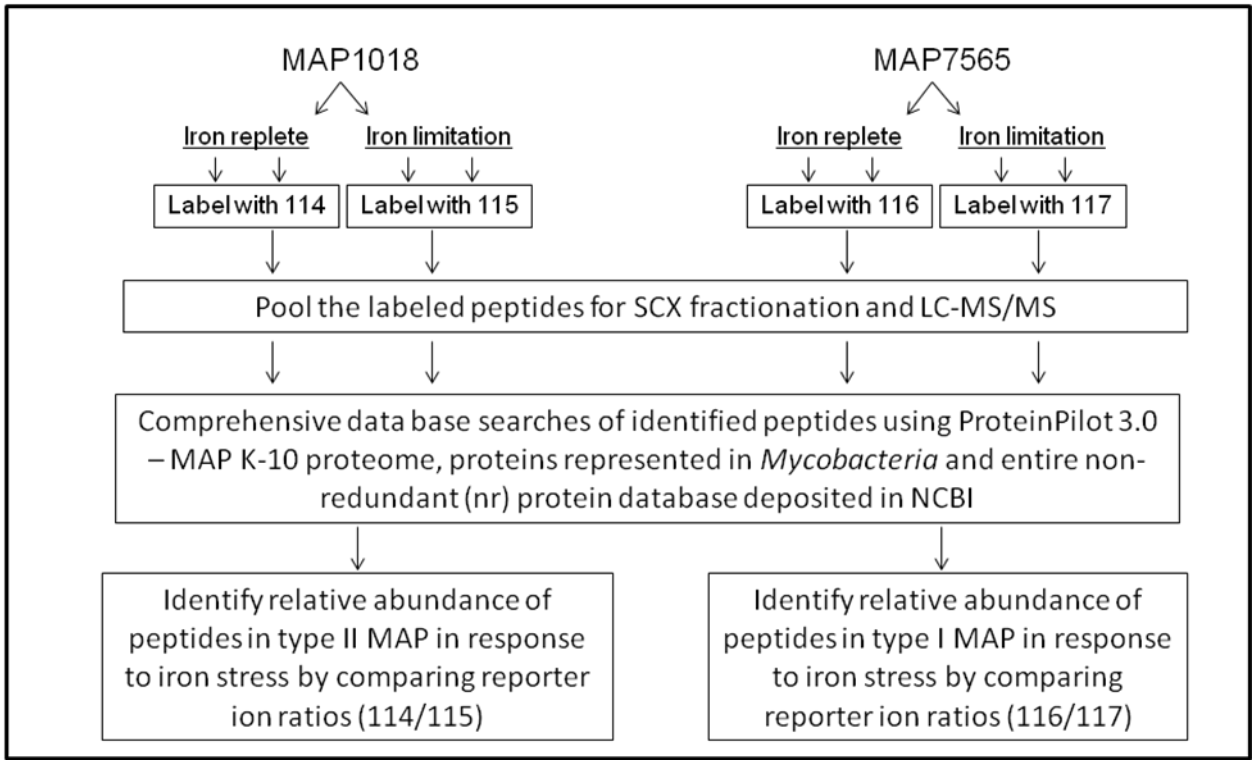

Figure S3: Shown here is the experimental design for proteomic profiling of the two type strains of MAP in response to iron. iTRAQ experiments were performed as described in methods section.

Figure S4: Iron repressed proteins similarly regulated between cattle and sheep MAP strains

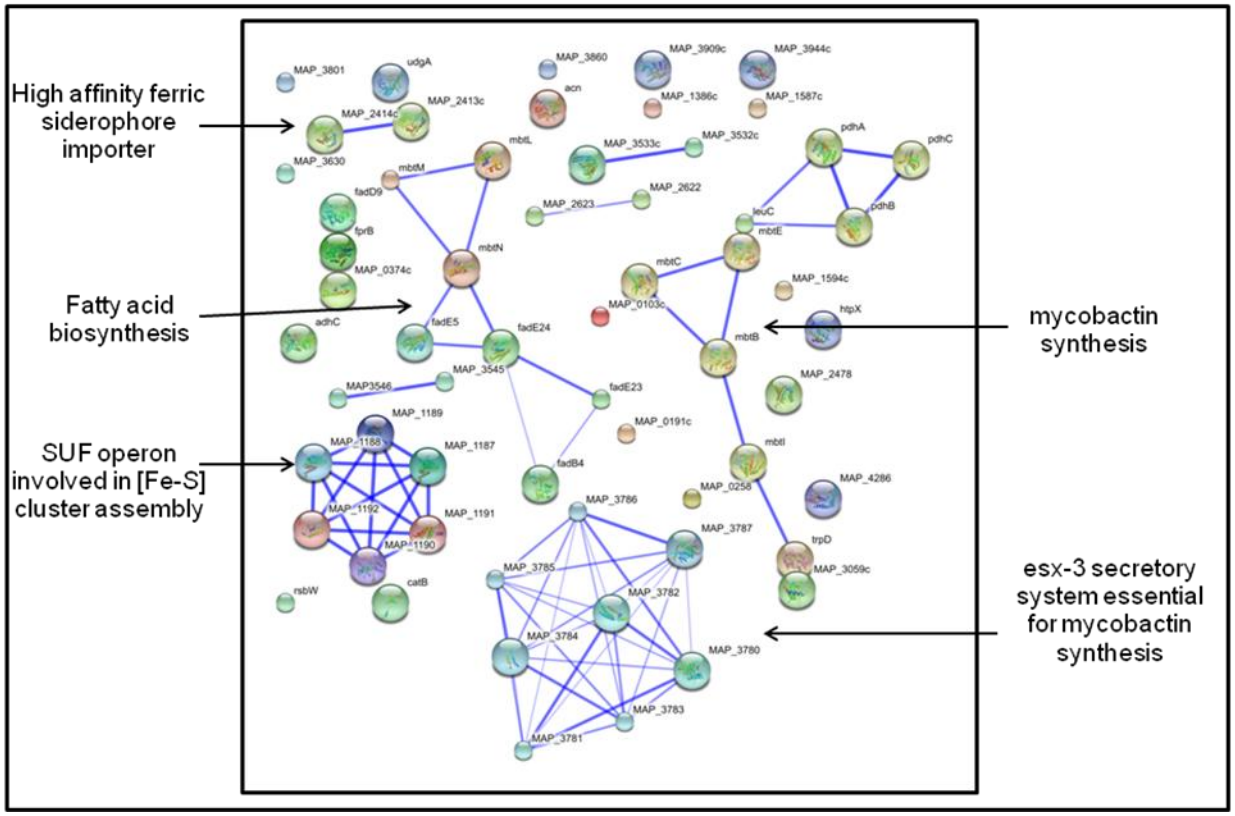

Figure S4: Shown here is the protein interaction network of proteins repressed under iron-replete conditions in both the type strains of MAP. Transcripts belonging to these proteins were also repressed under iron-replete conditions as observed by microarrays. Pathways were created using string database.

Figure S5: Proteins upregulated in the presence of iron by cattle MAP strain

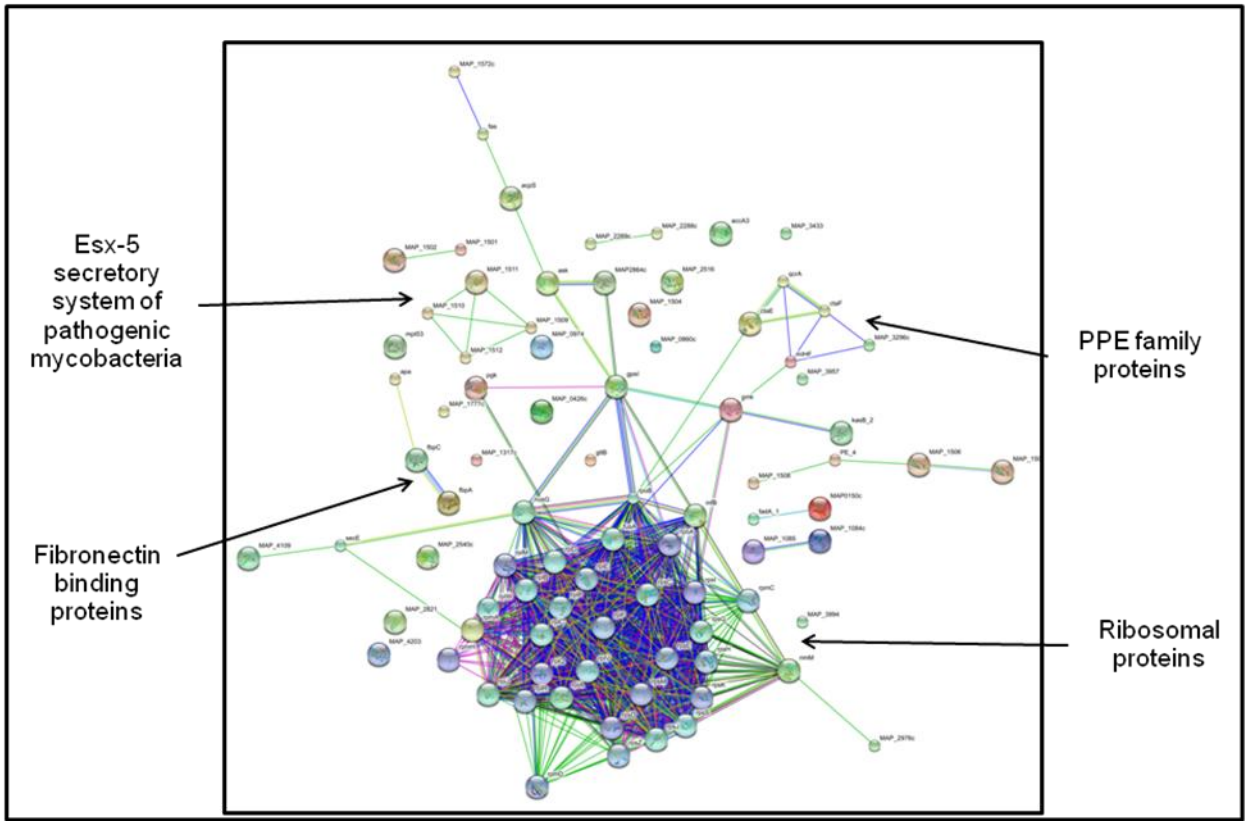

Figure S5: Shown here is the protein interaction network of proteins derepressed under iron-replete conditions in the cattle strain of MAP. Transcripts belonging to these proteins were also repressed under iron-replete conditions as observed by microarrays. Pathways were created using string database.