THE INFLUENCE OF Rel_{Mtb} ON THE EXPRESSION OF IMMUNOREGULATORY ANTIGENS IN *Mycobacterium tuberculosis*

DALIA LAU-BONILLA

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY

School of Molecular Biosciences

DECEMBER 2008

To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of DALIA LAU-BONILLA find it satisfactory and recommend that it be accepted.

_____

(Chair)

_____

_____

_____

_____

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mother for her unconditional love and support, as well to my sisters, brothers and their kids for always being there to remind me how blessed I am to have them in my life.

I also want to thank the members of the Dahl Lab: Dave Dutton, Dr. Kriti Arora and Dr. Danelle Whiteford for all the science discussions, but more importantly for the literary chats, coffee breaks, travels, life lessons and all the wonderful moments that made these past five and a half years, some of the best of my life.

I would like to thank my mentor, Dr. John L. Dahl, for his support and encouragement through all these years. I also want to thank my committee members, Dr. Mary Sanchez-Lanier, Dr. William Davis, Dr. John Nilson and Dr. Phil Mixter for their suggestions and advice. My gratitude also to Dr. Michael Konkel for his suggestions, guidance and support for the production of the rabbit antibodies.

I want to extend my thanks to the Fulbright program that made possible my dream of pursuing a doctoral degree by granting me a Fulbright scholarship. Its support during the first two years of my studies was the foundation of this success.

I would like to express my thanks to Kelly McGovern from the School of Molecular Biosciences for her invaluable help.

I would like to thank two very special people that unreservedly lent me their ears, and provided me with the emotional support and the tools to accomplish this goal. Omar and Patti without your help this would have been impossible.

To my friends Patti Elshafei, Kirstin Malm, and Anoria Haick thank you for all the great memories that you help me create. To my friends in Guatemala, especially

Rosario Hernandez, Teresita Jimenez, Sergio Lickes, Renata Pellecer, Patricia Ramirez, and Tamara Velasquez, thank you for not letting the distance or the time to change our friendship.

Finally, I just want to thank all family members, friends and Faculty at Washington State University and Universidad de San Carlos de Guatemala, that in any way contributed to the completion of this journey.

THE INFLUENCE OF Rel_{Mtb} ON THE EXPRESSION OF IMMUNOREGULATORY ANTIGENS IN *Mycobacterium tuberculosis*

ABSTRACT

by Dalia Lau-Bonilla, Ph.D.
Washington State University
December 2008

Chair: John L. Dahl

This study describes the effects of Rel_{Mtb} on the expression of *Mycobacterium tuberculosis* antigens. Rel_{Mtb} is a global regulator that coordinates the stringent response in *M. tuberculosis*. The main objective of this work was to find a correlation between the presence of Rel_{Mtb} and the expression of antigens that could modulate the immune response of the host. Finding this link will provide evidence that Rel_{Mtb} contributes to the pathogenesis and virulence of *M. tuberculosis*.

This work focused on two antigens that appear to be regulated by Rel_{Mtb}: Glutamine synthetase (GlnA1) and Antigen 84 ($Wag31_{Mtb}$). GlnA1 plays an essential role in nitrogen metabolism while $Wag31_{Mtb}$ participates in the process of cell division. The results presented here demonstrate that mycobacterial cells lacking Rel have a decreased production of both of these antigens. The lower concentrations of GlnA1 and $Wag31_{Mtb}$ could have an impact on intracellular survival, as well as an effect on the interactions of the mycobacterial cells with the immune mechanisms of the host. The work presented here provides preliminary data that can be used as the foundation to explore further: The

role of GlnA1 in the pathogenesis of *M. tuberculosis*, in particular on the production of P-L-glx and on the regulation of the intraphagosomal environment; and the potential of $Wag31_{Mtb}$ as an antigen and as inducer of phagocytosis. These features could be exploited in the development of vaccines and anti-tuberculosis drugs.

In conclusion, the findings presented here support the hypothesis that Rel_{Mtb} participates in the regulation of a wide variety of genes, including several involved in pathogenesis, antigenicity, and virulence. Accordingly, this work contributes to the field of mycobacteriology by providing more evidence of the significant role of Rel_{Mtb} in the physiology and pathogenesis of *M. tuberculosis*.

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

TABLE OF CONTENTS

DEDICATION

This dissertation is dedicated to my mother, Mrs. Zoila Carmelina Bonilla de Morales, and to the memory of my father, Roberto Alfonso Morales Lau.

CHAPTER ONE

INTRODUCTION

I. INTRODUCTION

More than a hundred species of the genus *Mycobacterium* have been identified to date (26). Most of these species are saprophytic dwellers of natural ecosystems in soil, water, and in the microbiota of higher organisms, including humans. Other species are opportunistic pathogens, that do not cause disease unless a pre-condition exists that compromises the immune system of the patient, such as HIV/AIDS, chemotherapy, or immunosuppression in organ-transplant individuals (64).

Only one third of the mycobacterial species identified so far are truly pathogens, capable of causing diseases in immunocompetent individuals. Out of this third, the members of the *Mycobacterium tuberculosis* complex and *Mycobaterium leprae* still represent a major challenge to public health around the world. *M. tuberculosis* alone claims almost three million lives each year (83). Other pathogenic species of mycobacteria that do not belong to the *M. tuberculosis* complex are called atypical mycobacteria or NTM (non-tuberculosis mycobacteria) (64).

In the following section, general characteristics of the genus *Mycobacterium* will be presented, as well as physiological and metabolic features of *M. tuberculosis* and *M. smegmatis*, the two species relevant to this research.

II. BACKGROUND INFORMATION

a. General characteristics of the genus *Mycobacterium*

The genus *Mycobacterium* is the only member of the family *Mycobacteriaceae*. This family belongs to the order of Actinomycetales comprising several genera of Gram-positive rods, the main characteristic of which is the presence of mycolic acids in their cell wall (39, 64). Mycolic acids are long chain α-alkyl, β-hydroxy fatty acids found in virtually all actinomycetes genera (9), however the amount present in mycobacterial cell walls is unusually high, accounting for 40 to 60% of the cell envelope dry weight (48).

Mycobacterial cells have a characteristic rod shape, usually straight or slightly curved. Most species of the genus are classified as non-motile, although recently it has been found that at least 2 species, *M. smegmatis* and *M. avium*, are able to carry out a type of motility known as sliding (19, 56). None of the species in the genus form spores, but they do have a unique cell envelope conformation. This cell envelope gives mycobacteria their ability to resist decoloration by acid-alcohol. For this reason mycobacteria are called acid-fast bacilli. This important feature is used to detect these microorganisms in clinical samples by the Ziehl-Neelsen staining technique, in which acid-fast organisms appear bright red against a blue background (39).

Another characteristic that sets mycobacteria apart, is their DNA G+C content; bacteria have a G+C from 25% to 75%, while all mycobacteria species have a high G+C content between 65-75% (except for *M. leprae*, 55%) (59). This trait is particularly

relevant when mycobacterial genes are inserted in other bacterial systems to be expressed. In the case of *Escherichia coli*, the G+C content is about 50% (59). Consequently, many mycobacterial genes are not expressed in *E. coli*, either because of codon bias or differences in promoters (41, 51).

The growth conditions required for most mycobacteria include temperatures between 35 and 37°C and a neutral pH. An atmosphere of 5 to 10% CO_2 is not required but if provided it would stimulate the growth of these organisms (39). The growth rates within the genus vary considerably depending on the species, but in comparison to most bacteria, all mycobacteria grow slowly. However, all the species in the genus can be separated into rapid growers, that require less than 7 days to generate visible colonies on solid media, and slow growers which require more than 7 days to give visible colonies (39, 64). *M. smegmatis* is considered a rapid grower, taking three to four days to produce visible colonies. *M. tuberculosis* on the other hand, is a slow grower requiring about three weeks to generate visible colonies.

b. Mycobacterial cell envelope

The cell envelope of mycobacteria is one of the most distinctive aspects of these organisms. It can be divided into three major structures: cell membrane, cell wall, and capsule (23)(Figure 1.1). The cell membrane is similar to those found in other Gram positive organisms, although it contains large amounts of phosphatidylinositol mannosides, a characteristic compound found in all actinomycetes (9).

As in many other bacteria, the core molecule of the cell wall is peptidoglycan, a polymer consisting of alternate layers of N-acetylglucosamine and N-acetylmuramic acid. This lattice arrangement provides mechanical support to the bacterial cell (82). Mycobacterial peptidoglycan is slightly different from other bacteria in that the muramic acid is glycosylated instead of acetylated, and in the presence of unusual cross-linking between chains (23, 69). Linked to peptidoglycan by esterified glycolipids linkages is arabinogalactan, a polysaccharide made of D-arabinofuranosyl and D-galactofuranosyl molecules. Another characteristic of all mycobacterial species is the presence of mycolic acids, which are bound to peptidoglycan by phosphodiester bridges. The basic structure of all mycolic acids is a long chain of α-branched-β-hydroxy fatty acids that may contain 60 to 90 carbon atoms, depending on the species of mycobacteria (4).

The complex formed by the covalently bound peptidoglycan, arabinogalactan, and mycolic acids is known as the cell wall skeleton. This structure plays a significant role in the resistance of mycobacterial cells to the action of lytic enzymes produced by infected hosts and for providing a selective permeability to the organism (23).

In stationary, undisturbed liquid cultures, there is an assortment of proteins and polysaccharides that forms a capsule surrounding the mycobacterial cell. This capsule is composed mainly of a glycogen-type glucan, arabinomannan, mannan, proteins, and a few lipids. This structure is thought to be shed and replaced continuously during the different growth stages of mycobacterial cells (23, 84). The capsule contributes to the permeability barrier and to some degree, facilitates the survival of pathogenic mycobacteria inside macrophages (22).

In addition to these major components, other important molecules are found attached to the cell envelope, namely lipoarabinomannan (LAM), lipoolygosaccharides (LOS), phenolic glycolipids (PGL's), glycopeptidolipids (GPL's), acetylated trehaloses, phospholipids, and sulpholipids. These lipids are not covalently linked to the cell envelope and therefore are referred to as free lipids (47). The quantity and type of these components are specific for each mycobacterial species. Some of them have strong antigenic properties (i.e. LAM), play an important role in the pathogenesis of mycobacterioses, and affect the morphological appearance of mycobacterial colonies (10, 64). *M. tuberculosis* produces large amounts of trehalose-6-6-dimycolate, also known as cord factor, which has immunomodulatory activities (40, 64).

The composition and arrangement of the cell wall skeleton and capsule provide an excellent permeability barrier to mycobacteria. However, this barrier could also pose a disadvantage in terms of nutrient acquisition. To overcome this problem, mycobacteria have porins located in the cell wall skeleton (61, 62). These proteins are specialized in the transport of small hydrophilic molecules. Mycobacterial cell walls have a very low permeability for hydrophobic solutes, these appear to diffuse through the different lipid layers in order to enter the cell. This low permeability accounts for the resistance of mycobacteria to several drugs and also confers the acid-fastness characteristic of these organisms (10, 22).

The complexities of mycobacterial cell walls represent a challenge to the cell division process, which in *M. tuberculosis* plays an important part in the pathogenesis of the organism (38).

c. Mycobacterial cell division

Mycobacterial species are slow growers in comparison with most bacteria cultivable in laboratory conditions. Still, mycobacterial species can be separated into two groups: rapid growers, and slow growers. Rapid growers, such as *M. smegmatis,* have a doubling time of 2 to 3 hours; slow growers have doubling times ranging from 12 hours (i.e. *M. avium*) to 22- 24 hours (i.e. *M. tuberculosis*) and even more than 14 days (i.e. *M. leprae*) (65). Although the longer generation time of mycobacteria compared to other bacterial species is far from being fully understood, several hypotheses have been proposed to explain it. One hypothesis asserts that the low permeability of the mycobacterial cell envelope hinder the acquisition of nutrients, forcing the organisms to slow down their growth (64). This idea is supported by experiments in which porins from a rapid grower *Mycobacterium* were expressed in slow grower species, resulting in shorter generation times by the latter (54). Other hypotheses include slow RNA synthesis, slow DNA elongation, and slow protein synthesis (64, 77), all of which increase the generation time of the cells.

Bacterial cell division is a highly organized process in which DNA replication, septum formation, cell wall synthesis, and ultimately cell partition do not occur in sequence but rather in an overlapping manner (33). Cell division has been widely studied in *Bacillus subtilis* and *E. coli,* and most of the events that occur in these bacteria could be extrapolated to mycobacteria. However, homologs of several genes that play a role in cell division in *B. subtilis* and *E. coli* seem to be absent from the mycobacteria species whose genomes have been fully annotated (65). This suggests that other unidentified

genes may participate in mycobacterial cell division. Among mycobacterial genes that have homologs in other bacteria, *ftsZ* is one of the better characterized (25, 33). FtsZ initiates the process of cell division by attaching to the membrane in the center of the cell and polymerizing to form a circular structure known as the Z-ring. This formation is subsequently stabilized by FtsW (21, 65). In addition to stabilizing the Z-ring, FtsW transports peptidoglycan precursors and recruits FtsI to the division site. FtsI is a penicillin-binding protein required for the synthesis of peptidoglycan and septum formation (25). Once the septum has been completed and the two daughter cells are clearly defined by an independent cell wall, peptidoglycan hydrolases digest the remaining septal material, resulting in the release of the two daughter cells (38). The assembly of the Z-ring is negatively regulated in mycobacteria by Wag31 (also known as DivIVA or Antigen 84). Wag 31 is a highly conserved protein that localizes to the poles of the dividing cell, this prevents septum formation at the poles and forces the Z-ring to localize at the center of the cell (38). Regulation of cell growth and cell division in mycobacteria occurs by reversible phosphorylation through the action of two serine/threonine protein kinases, PknA and PknB (45). These two proteins are part of the Rv0014c-Rv0018c operon in *M. tuberculosis* and are predominantly expressed during logarithmic growth (45). PknA phosphorylates FtsZ, resulting in a decrease in the polymerization of FtsZ and inhibition of the Z-ring formation (78). PknB is responsible for the phosphorylation of several substrates, including a penicillin-binding protein required for peptidoglycan synthesis at the septum (20). It is in a phosphorylated state that Wag31 is active and localizes to the cell poles as described above (38, 45).

d. *Mycobacterium smegmatis*

M. smegmatis is a rapid growing species of mycobacteria. In comparison with *M. tuberculosis*, *M. smegmatis* grows about 10 times faster, it produces visible colonies in 3 to 4 days. *M. smegmatis* was considered an environmental saprophytic species until two decades ago, when it was isolated from a lung biopsy. Since then, *M. smegmatis* has been reported in rare cases of cellulitis, localized abscesses, osteomyelitis, wound infections, and other conditions (11, 46).

M. smegmatis plays a crucial role as a substitute host for genetic manipulation of mycobacterial genes from *M. tuberculosis*, *M. leprae* and other pathogenic, slow growing mycobacterial species (57). Many *M. tuberculosis* genes cannot be expressed in *E. coli* due to the difference in G+C content between *E. coli* (50%) and *M. tuberculosis* (65.6%) which causes codon usage bias (51). This problem can be overcome by the use of *M. smegmatis* in which the G+C content (67.4%) is more similar to that of *M. tuberculosis* (59). Post-translational modifications, such as glycosylation, of *M. tuberculosis* proteins are also accomplished by *M. smegmatis* but not by *E. coli* (37). Indeed, several studies have demonstrated that *M. tuberculosis* genes can be expressed in *M. smegmatis* (29, 79, 81). The main advantages of using *M. smegmatis* as a surrogate host for *M. tuberculosis* genes are that *M. smegmatis* is a fast grower, it produces visible colonies on solid media in 3 to 4 days as opposed to 3 to 4 weeks as occurs in *M. tuberculosis*; furthermore, work with *M. smegmatis* can be carried out in biosafety level 2 laboratory conditions, instead of the rigorous containment conditions of biosafety level 3 necessary to work with *M. tuberculosis* (35).

e. *Mycobacterium tuberculosis*

Despite the fact that tuberculosis (TB) is one of the oldest recognized diseases afflicting humans, it is still a major public health problem worldwide. According to the World Health Organization (WHO), it is estimated that 2 billion people around the Globe are infected with one species of the *M. tuberculosis* complex, the causative agents of TB. Out of this staggering number, almost three million people die of TB each year, and 8 million people develop an active infection in the same period (83).

Although the vast majority of TB cases can be treated with drugs that have been available since the 1940's, efforts to eradicate the disease have been unsuccessful and the global situation has not improved. One reason for this has been the rise of the Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) pandemic in the last two decades. As the number of immunocompromised people has increased, there are a greater number of people converting from latent TB infection to developing the active disease. There are numerous hurdles to reducing levels of TB that include: low sensitivity of diagnostic tools, lack of an effective vaccine, poor adherence to a drug treatment that requires at least 6 months to be effective, and widespread occurrence of multi-drug resistant *M. tuberculosis* strains (83).

The *Mycobacterium tuberculosis* complex comprises seven species of very closely related mycobacteria: *M. tuberculosis, M. bovis, M. caprae, M. pinipedii, M. canettii, M. africanicum*, and *M. microti.* Although all of these species are able to cause TB in a variety of hosts, *M. tuberculosis* accounts for most of the infections in humans.

Due to their ability to withstand the effect of decoloration with acid-alcohol, mycobacteria are called acid-fast bacilli. The detection of acid-fast rods in clinical samples constitutes the most widespread method of diagnosis of TB. However, the acid-fastness of the cells varies depending on the growth stage of the organism, and the test requires an estimated 10^4 rods/ml of sample to give a reliable result (14, 39). Another tool used to detect TB is the culture of the organism from clinical samples. However, the development of visible colonies of Mtb in solid media takes from 2 to 3 weeks, and this lowers the value of this technique as a diagnostic tool. Although advances in molecular techniques have allowed the development of DNA-based diagnostic tests, they haven't been adopted extensively in developing countries, where the majority of TB cases occur (14).

The current treatment of TB involves four drugs, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB). The drugs are administered for a period of at least 6 months divided into two phases. The initial intensive phase usually comprises the first 2 months, during which the four drugs are given together daily. This is followed by a continuation phase that lasts 4 months, in which only RIF and INH are provided (63). The goal of this plan is to kill active replicating bacteria in the first two months; in the continuation phase, RIF is aimed at killing any remaining dormant bacteria and INH prevents the emergence on RIF-resistant bacilli (63, 64, 83). Multiple drugs are given at the same time to prevent the growth of bacteria resistant to an individual drug.

The development of drug resistance in *M. tuberculosis* strains is a problem that has hindered the progress in the control of TB since the first anti-TB drug, streptomycin,

was used more than 60 years ago. Natural mutations occur in the genome of *M. tuberculosis* that produce resistance to a particular drug in a frequency of 10^{-6} to 10^{-8} (16). The application of mono-therapy, the lack of adherence to the 6 month period of the multi-drug therapy, or the intermittent administration of the drugs, all exacerbate the selection of mutant strains resistant to one or more of the currently available drugs (83).

e.1. Pathogenesis and Immunology of Tuberculosis

The most common route of infection for TB is by inhalation (64). Upon entering the respiratory system, bacilli are phagocytized by alveolar macrophages. From this point the fate of the infected bacilli will depend on three main factors: the infectious dose, the virulence of the infective strain, and the immune response of the host. Two scenarios can occur: the ingested bacilli are killed by the macrophages, thus preventing the development of TB; or the ingested bacilli are able to survive inside phagosomes in the macrophages, replicate, and eventually kill the macrophage. The newly released bacteria are ingested by other immature macrophages, in a continuous cycle of phagocytosis, multiplication of bacilli, destruction of the infected macrophage, and release of bacteria in the alveolar environment (70). This is considered the primary lesion in TB, and is formed by concentric layers of immature macrophages infected with *M. tuberculosis*. During this stage, there seems to be no control by the host over the mycobacterial growth, allowing dissemination of *M. tuberculosis* through the bloodstream, and the spread of the pathogen to the spleen, liver and other organs. Cell-mediated immunity against *M. tuberculosis* develops within 2 to 6 weeks after infection.

Infected macrophages release IFN-γ and TNF-α to recruit and activate fibroblasts and direct them to the early lesion. The production of IL-12 by macrophages also leads to the development of a Th1 response, with an increased production of IFN-γ (68). IFN-γ is a major activator of macrophages and it is crucial in the response against TB (28). TNF-α induces chemokines that in turn direct granuloma formation. A related function of TNF-α is to maintain the structural integrity of the granulomas by continuously recruiting macrophages (74).

The immune response triggered by *M. tuberculosis* elicits seemingly contradicting cytokine reactions. Although the induction of pro-inflammatory cytokines is justified for their role in granuloma formation and maintenance, the reason for inducing anti-inflammatory cytokines like IL-4 and IL-10 remains obscure (1, 28, 68)

The continuous activation and lysis of macrophages, eventually leads to the formation of granulomas, a signature characteristic of TB. As the infected macrophages lyse, a liquefied or caseous material is produced. This material is rich in *M. tuberculosis* cells ready to infect new macrophages. In order to prevent the infection from disseminating further, mature macrophages surround the caseous lesion, forming granulomas, structures that contain and isolate *M. tuberculosis* from the rest of the lung tissue. The environment inside the granuloma is thought to be low in nutrients and oxygen, which forces the multiplication of *M. tuberculosis* cells to slow down and eventually to stop. *M. tuberculosis* cells then enter a latency or dormancy stage that could last several months or decades in immunocompetent individuals. This dormant

state is characterized by a very low metabolic rate in the bacilli and will be discussed in a separate section (15).

Any condition that triggers immunosupression (stress, malnutrition, chemotherapy, HIV infection, etc) could lead to the reactivation of the dormant bacilli and the development of the disease. The *M. tuberculosis* cells resume multiplication, leading to the liquefaction of the center of the lesion. The granuloma ultimately breaks open, releasing the liquefied material into near bronchi and leaving an empty cavity behind. These cavities are easily seen by radiography and constitute the hallmark of chronic TB. The newly released *M. tuberculosis* could then be expelled from the lungs by coughing and talking, allowing them to reach other hosts. If left untreated, the disease progresses causing necrosis and other damage to lungs and other organs, leading to the death of the patient (70).

e.2. *M. tuberculosis* antigens and their role in the modulation of the host immune response

M. tuberculosis cells are highly immunogenic. Several proteins, lipids and carbohydrates that are part of *M. tuberculosis* cells have shown antigenic activity (70). Some *M. tuberculosis* antigens of interest, due to their potential use as diagnostic markers and in the development of new vaccines, are the 85 kDa antigen complex, 88 kDa, 65kDa, 45kDa, 30/31kDa, 19kD antigens and the 38 kDa lipoprotein (49, 52, 71, 72, 73). Also important are the low molecular mass antigens between 12-14 kDa, GroES (Barnes 1991), ESAT-6 (76), CFP10 (7). Non-protein antigens that have been explored

include 2,3 diacyl trehalose (DAT), lipooligosaccharides (LOS) and trehalose dicarboxylic acid bis N, N-dioctadecylamide (BDA-TDA) (44).

The immunomodulatory properties of some *M. tuberculosis* antigens corroborate the hypothesis that *M. tuberculosis* manipulates the host immune response to its advantage (17, 30). For instance, the 19kDa lipoprotein, recognized as a strong antigen, inhibits the production of IFN-γ and antigen processing by MHC-II, which in turn could allow *M. tuberculosis* to evade effector and memory $CD4^+$ T cells responses (66). Another example is the lipoarabinomannan (LAM) produced by highly virulent strains of *M. tuberculosis* seems to inhibit the production of TNF-α, IFN-α and nitric oxide in infected macrophages, contributing to the survival of the bacilli inside these macrophages (24, 55). In the case of LAM and other components of the cell envelope even small changes, such as acylation, in the composition of lipids and lipopolysaccharides, could help *M. tuberculosis* to escape host immune defenses by manipulating the type of cytokines produced (32).

M. tuberculosis antigens also modulate the expression of chemokines like monocyte chemo-attractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), and the expression of the chemokines receptors CCR2 and CCR5 (2). The interaction of monocytes and macrophages with these molecules facilitates the formation of granulomas by recruiting cells to the site of infection (2, 3, 58).

Relevant to this work are glutamine synthetase 1 (GlnA1) and $Wag31_{Mtb}$. GlnA1 is a highly immunogenic protein, found in large concentrations in the culture filtrate of *M.*

tuberculosis (80). It plays an essential role in nitrogen metabolism and is thought to be involved in inhibiting acidification in phagosomes containing *M. tuberculosis* (34). $Wag31_{Mtb}$, also known as Antigen 84, is also a strong immunogenic protein, with important roles in cell wall biosynthesis and cell division (36, 60).

f. Stringent Response

Bacterial cells facing adverse conditions, such as lack of nutrients or environmental stresses, activate a global regulatory system known as the stringent response. Originally described in *E. coli* as a response to amino acid starvation (12) the stringent response has been found in many bacteria and even in plants (8). Moreover, it has been demonstrated that the stringent response is activated not only by amino acid depletion but by a large array of environmental changes (8, 13).

The stringent response is characterized by a rapid accumulation of guanosine 3'-diphosphate,5'-triphosphate and guanosine 3',5'-bispyrophosphate, both referred to as (p)ppGpp; and by the down regulation of stable RNA synthesis that results in growth arrest (8, 12). In *E. coli* and other Gram-negative organisms two proteins are responsible for the regulation of the stringent response: RelA and SpoT (12, 42, 43, 53). RelA is a (p)ppGpp synthetase that gets activated by uncharged tRNA that accumulates as a result of amino acid deprivation (13). SpoT functions both as a synthetase and as a hydrolase, regulating the amount of accumulated (p)ppGpp. Although the specific activator of SpoT has not been identified, its activity is thought to be triggered by stresses other than amino acid starvation (12, 53).

During amino acid starvation, (p)ppGpp synthesized by RelA binds the β-subunit of RNA polymerase, this hinders RNA polymerase activity, causing a decrease in the synthesis of stable RNA and inhibiting activities such as DNA replication, and synthesis of fatty acids and other cell wall components. On the other hand, stress resistance proteins and starvation survival mechanisms are activated and enhanced (8, 53). Traditionally the stringent response has been linked to the stationary phase of bacterial growth, however there is evidence that RelA/SpoT and (p)ppGpp are involved in other events in the growth and physiology of several microorganisms (53). For example, it has been shown that the stringent response plays an important role in the pathogenesis and virulence of several bacteria (31).

In mycobacteria a single homologue for both RelA and SpoT has been identified. In *M. tuberculosis*, the Rel protein (Rel_{Mtb}) is comprised of 738 amino acids and functions as ATP:GTP/GDP/ITP 3'-pyrophoisphoryltransferase and a (p)ppGpp 3'-pyrophosphorylhydrolase (5). The role of Rel_{Mtb} in the virulence and long term survival of *M. tuberculosis* has been investigated *in vitro* and *in vivo* (18, 67). A *rel* knock out strain of *M. tuberculosis* (H37RvΔrel_{Mtb}) was unable to accumulate (p)ppGpp when exposed to nutrient starvation in a buffer solution without nutrients, as opposed to the wild type strain (H37Rv) that began accumulation of (p)ppGpp after only 20 min of exposure to this starvation conditions (67). In the same study, H37RvΔrel_{Mtb} grew consistently slower than H37Rv when growing in regular media, suggesting that the activity of Rel_{Mtb} is necessary even when nutrients and other conditions for optimal growth are provided (67). Experiments carried out in mice revealed that H37RvΔrel_{Mtb} was unable to sustain a chronic infection, and had a severely reduced ability to induce

granuloma formation, providing evidence that Rel_{Mtb} plays an important role in the pathogenesis of TB (18). Furthermore, microarray analysis comparing H37Rv and H37RvΔrel_{Mtb} under nutrient starvation conditions has shown that more than 150 genes seem to be under the regulation of Rel_{Mtb}, these include several antigens, genes involved in virulence, persistence, and cell wall synthesis (18). These findings highlight the importance of granulomas in TB and the role of Rel_{Mtb} in the survival and persistence of *M. tuberculosis* inside them. Although a granuloma is a mechanism of the host to contain and control an infection, it also provides a safe haven for *M. tuberculosis*, where the bacilli can remain in a dormant state for years (15). The lack of Rel in H37RvΔrel_{Mtb} not just deprives the pathogen from these refuges but also has deleterious effects on the bacilli that do manage to be encapsulated in one. Inside granulomas *M. tuberculosis* cells do not have access to nutrients and therefore, need to activate the stringent response in order to enter dormancy. H37RvΔrel_{Mtb} cells are unable to activate the stringent response, and to enter dormancy, this eventually leads to death of the bacilli and clearing of the infection (18).

III. CONCLUDING REMARKS

The work presented in this dissertation encompasses the research done on two *M. tuberculosis* antigens that appear to be under the control of Rel_{Mtb}: GlnA1 and Wag31. Both of these antigens appeared down regulated in H37RvΔ*rel*$_{Mtb}$ in comparison with H37Rv when cells were grown in normal conditions.

The central hypothesis addressed here is that Rel_{Mtb} regulates the expression of *M. tuberculosis* antigens that participate in the manipulation of the immune response.

The significance of this work is that it provides more evidence of the role of Rel_{Mtb} on the expression of important *M. tuberculosis* antigens, and as such, it plays a significant part in the pathogenesis of TB.

In the next pages, the work done on GlnA1 and $Wag31_{Mtb}$ is presented in two individual chapters. A final chapter includes a summary of the most important findings in this work, a discussion of the significance of those findings, and expands in the future directions that can be taken using the information presented here.

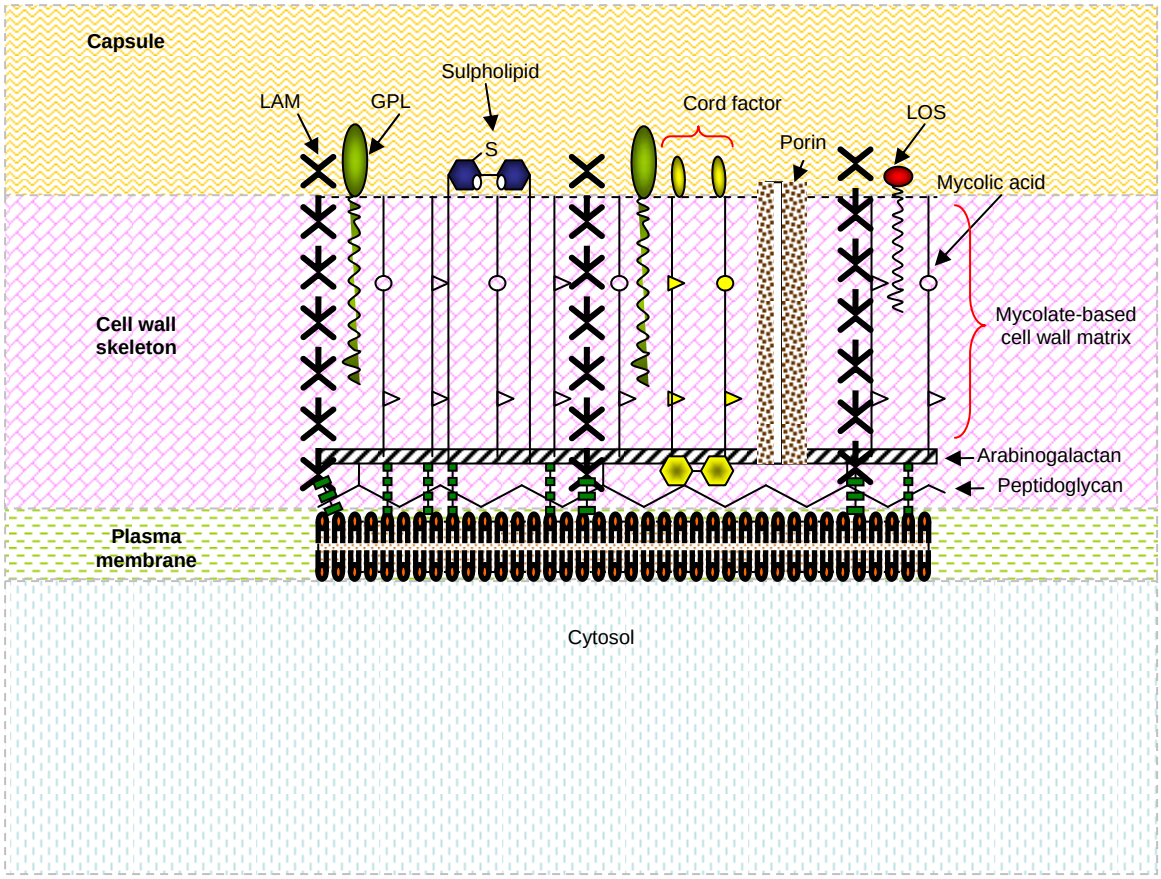

Figure 1.1. **Mycobacterial cell envelope**. The three major fractions of the cell envelope are delimited by different colors. Plasma membrane (light green); cell wall skeleton (pink) includes peptidoglycan, arabinogalactan, and mycolic acids; capsule (light yellow) includes free lipids: liporarabinomannan (LAM), glycopeptidolipis (GPL's), sulpholipids, trehalose-6-6-dimycolate (cord factor), and lipooligosaccharides (LOS).
Modified from Brennan P.J. 1988 and Daffe M et al 1997

IV. REFERENCES

1. **Andersen, P., D. Askgaard, L. Ljungqvist, M. Weis Bentzon, I. Heron.** 1991. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. *Infect. Immun.* **59**(4):1558-1563.

2. **Arias, M. A., A. E. Pantoja, G. Jaramillo, S. C. Paris, R. J. Shattock, L. F. Garcia, G. E. Griffin.** 2006. Chemokine receptor expression and modulation by *Mycobacterium tuberculosis* antigens on mononuclear cells from human lymphoid tissues. *Immunology*. **118**:171-184.

3. **Arias, M. A., G. Jaramillo, Y. P. Lopez, N. Mejia, C. Mejia, A. E. Pantoja, R. J. Shattock, L. F. Garcia, G. E. Griffin.** 2007. *Mycobacterium tuberculosis* antigens specifically modulate CCR2 and MCP-1/CCL2 on lymphoid cells from human pulmonary hilar lymph nodes. *J. Immunol.* **179**:8381-8391.

4. **Asselineau C., J. Asselineau, G. Laneelle, M. A. Laneelle.** 2002. The biosynthesis of mycolic acids by Mycobacteria: current and alternative hypotheses. *Prog. Lipid Res.* **41**:501-523.

5. **Avarock D., J. Salem, L. Li, Z. Wang, H. Rubin.** 1999. Cloning and characterization of a bifunctional RelA/SpoT homologue from *Mycobacterium tuberculosis*. *Gene* **233**:261-269.

6. **Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. *J. Immunol.* **149**(2):541-547.

7. **Berthet F. X., P. B. Rasmussen, I. Rosenkrands, P. Andersen, B. Gicquel.** 1998. A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). *Microbiology*. **144**:3195-3203.

8. **Braeken, K., M. Morris, R. Daniels, J. Vanderleyden, J. Michiels.** 2006. New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol* **14**:45-54.

9. Brennan, P. J. 1988. Mycobacterium and other actinomycetes. In *Microbial Lipids.* Vol. 1, Eds. C. Ratledge and S.G. Wilkinson, Academic Press. London, pp. 203-298.

10. **Brennan, P. J.** 1995. The envelope of mycobacteria. *Annu. Rev. Biochem.* **64**:29-63.

11. **Brown-Elliott, B. A., R. J. Wallace, Jr.** 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin. Microbiol. Rev.* **15**(4):716-746.

12. **Cashel, M., D. R. Gentry, V. J. Hernandez, D. Vinella.** 1987. The stringent response. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. ASM Press.

13. **Chatterji, D., A. K. Ojha.** 2001. Revisiting the stringent response, ppGpp and starvation signaling. *Curr. Opin. Microbiol.* **4:**160-165.

14. **Cheng, V. C. C., W. W. Yew., K. Y. Yuen.** 2005. Molecular diagnostics in tuberculosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **24**:711-720.

15. **Collins, H. L., S. H. E. Kaufmann.** 2001. The many faces of host responses to tuberculosis. *Immunology*. **103:**1-9.

16. **Connell N, Belanger A.** 2000. Mutants and Mutagenesis *in Molecular Genetics of Mycobacteria* Edited by G. F. Hatfull, W. R. Jacobs. ASM Press, Washington D.C.

17. **Daffe, M., P. Draper.** 1998. The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol.* **39**:131-203.

18. **Dahl, J. L., C. N. Kraus, H. I. M. Boshoff, B. Doan, K. Foley, D. Avarock, G. Kaplan, V. Mizrahi, H. Rubin, C. E. Barry III.** 2003. The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc. Natl. Acad. Sci. USA*. **100**:10026-10031.

19. **Dahl, J.L., K. Arora, H. I. Boshoff, D. C. Whiteford, S. A. Pacheco, O. J. Walsh, D. Lau-Bonilla, W. B. Davis, A. G. Garza.** 2005. The *relA* homolog of *Mycobacterium smegmatis* affects cell appearance, viability, and gene expression. *J. Bacteriol.* **187**:2439-2447

20. **Dasgupta, A., P. Datta, M. Kundu, J. Basu.** 2006. The serine/threonine kinase PknB of *Mycobacterium tuberculosis* Phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology*. **152**:493-504.

21. **Datta, P., A. Dasgupta, S. Bhakta, J. Basu.** 2002. Interaction between FtsZ and FtsW of *Mycobacterium tuberculosis*. *J. Biol. Chem*. **277**(28):24983-24987.

22. **Draper, P.** 1998. The outer parts of the mycobacterial envelope as permeability barriers. *Front. Biosci.***3**:D1253-1261.

23. **Draper, P. M. Daffe.** 2005. The cell envelope of *Mycobacterium tuberculosis* with special reference to the capsule and outer permeability barrier. In *Tuberculosis and the tubercle bacillus.* Cole, S.T., K. D. Eisenach, D. N. McMurray, W. R. Jacobs, Jr. eds. Washington DC. *ASM Press.*

24. **Ellner, J. J., T. M. Daniel.** 1979. Immunosuppression by mycobacterial arabinomannan. *Clin exp. Immunol.* **35**:250-257.

25. **Errington J., R. A. Daniel, D. Scheffers.** 2003. Cytokinesis in bacteria. *Microbiol. Mol. Biol. Rev.* **67**(1):52-65.

26. **Euzeby, J.P.** 2008. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *Int. J. Syst. Bacteriol.*, 1997, **47**, 590-592. (List of Prokaryotic names with Standing in Nomenclature. Last full update: November 05, 2008. URL: http://www.bacterio.net).

27. **Faller, M., M. Niederweis, G. E. Schulz.** 2004. The structure of a mycobacterial outer-membrane channel. *Science.* **303**:1189-1192.

28. **Flynn, J. L., J. Chan.** 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* **19**:93-129.

29. **Garbe, T., D. Harris, M. Vordermeier, R. Lathigra, J. Ivanyi, D. Young.** 1993. Expression of the *mycobacterium tuberculosis* 19-Kilodalton antigen in *mycobacterium smegmatis*: immunological analysis and evidence of glycosylation. *Infect. Immun.* **61**(1):260-267.

30. **Gehring, A. J., K. M. Dobos, J. T. Belisle, C. V. Harding, W. H. Boom.** 2004. *Mycobacterium tuberculosis* LprG (*Rv1411c*): A novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J. Immunol.* **173**:2660-2668.

31. **Godfrey, H. P., J. V. Bugrysheva, F. C. Cabello.** 2002. The role of the stringent response in the pathogenesis of bacterial infections. *Trends Microbiol.* **10**(8):349-351.

32. **Gilleron, M., J. Nigou, D. Nicolle, V. Quesniaux, G. Puzo.** 2006. The acylation state of mycobacterial lipomannans modulates innate immunity response through Toll-like receptor 2. *Chem. Biol.* **13**:39-47.

33. **Haeusser, D. P., P. A. Levin.** 2008. The great divide: coordinating cell cycle events during bacterial growth and division. *Curr. Opin. Microbiol.* **11**:94-99.

34. **Harth, G., D. L. Clemens, M. A. Horwitz.** 1994. Glutamine synthetase of *Mycobacterium tuberculosis*: Extracellular release and characterization of its enzymatic activity. *Proc. Natl. Acad. Sci. USA.* **91**:9342-9346.

35. **Hatfull, G. F., W. R. Jacobs Jr.,** Editors**.** 2000. Molecular genetics of mycobacteria. *ASM Press.*

36. **Hermans, P. W. M., F. Abebe, V. I. O. Kuteyi, A. H. J. Kolk, J. E. Thole, M. Harboe.** 1995. Molecular and Immunological characterization of the highly conserved antigen 84 from *Mycobacterium tuberculosis* and *Mycobacterium leprae. Infect. Immun.* **63**(3):954-960.

37. **Herrmann, J. L., R. Delahay, A. Gallagher, B. Robertson, D. Young.** 2000. Analysis of post-translational modification of mycobacterial proteins using a cassette expression system. *FEBS Lett.* **473**:358-362.

38. **Hett, E. C., E. J. Rubin.** 2008. Bacterial growth and cell division: a mycobacterial perspective. *Microbiol. Mol. Biol. Rev*. **72**(1):126-156.

39.Inderlied, C. B., G. E. Pfyffer. 2003. Susceptibility test methods: *Mycobacteria*. In *Manual of Clinical Microbiology*, 8th ed., Washington, DC: *ASM Press*.

40. **Indrigo, J., R. L. Hunter, Jr., J. K. Actor.** 2002. Influence of trehalose 6,6'-dimycolate (TDM) during mycobacterial infection of bone marrow macrophages. *Microbiology*. **148**:1991-1998.

41. **Jacobs, Jr. R. W.** 2000. *Mycobacterium tuberculosis*: A once genetically intractable organism. In *Molecular genetics of mycobacteria*. Eds. Hatfull, G. F. and Jacobs Jr, W. R. *ASM Press.*

42. **Jain , V., M.. Kumar, D. Chatterji.** 2006. ppGpp: Stringent response and survival. *J. Microbiol.* **44**(1):1-10.

43. **Jain, V. R. Saleem-Batcha, A. China, D. Chatterji.** 2006. Molecular dissection of the mycobacterial stringent response protein Rel. *Prot. Sci.* **15**:1449-1464.

44. **Julian, E., L. Matas, J. Alcaide, M. Luquin.** 2004. Comparison of antibody responses to a potential combination of specific glycolipids and proteins for test sensitivity improvement in tuberculosis serodiagnosis. *Clin. Diagn. Lab. Immun.* **11**(1):70-76.

45. **Kang, C., D. W. Abbott, S. T. Park, C. C. Dascher, L. C. Cantley, R. N. Husson.** 2005. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. *Genes Dev.* **19**:1692-1704.

46. **Katoch, V. M.** 2004. Infections due to non-tuberculous mycobacteria (NTM). *Indian J. Med. Res.* **120**:290-304.

47. **Kremer, L., G. S. Besra.** 2005. A waxy tale, by *Mycobacterium tuberculosis.* In *Tuberculosis and the tubercle bacillus.* Cole, S.T., K. D. Eisenach, D. N. McMurray, W. R. Jacobs, Jr. eds. Washington DC. *ASM Press.*

48. **Korf, J., A. Stoltz, J. Verschoor, P. De Baetselier, J. Grooten.** 2005. The 2005. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *Eur. J. Immunol.* **35**:890-900.

49. **Laal, S., K. M. Samanich, M. G. Sonnenberg, S. Zolla-Pazner, J.M. Phadtare, J. T. Belisle.** 1997. Human humoral responses to antigens of *Mycobacterium tuberculosis*: Immunodominance of high-molecular-mass antigens. *Clin. Diagn. Lab. Immunol.* **4**(1):49-56.

50. **Laal, S., K. M. Samanich, M. G. Sonnenberg, J. T. Belisle, J. O'Leary, M. S. Simberkoff, S. Zolla-Pazner.** 1997. Surrogate marker of preclinical tuberculosis in human immunodeficiency virus infection: Antibodies to a 88-kDa secreted antigen of *Mycobacterium tuberculosis*. *J. Infect. Dis.* **176**(1):133-142.

51. **Lakey, D. L., R. K. R. Voladri, K. M. Edwards, C. Hager, B. Samten, R. S. Wallis, P. F. Barnes, D. S. Kernodle.** 2000. Enhanced production of recombinant *Mycobacterium tuberculosis* antigens in *Escherichia coli* by replacement of low-usage codons. *Infect. Immun.* **68**(1):233-238.

52. **Lim, R. L. H., L. K. Tan, W. F. Lau, M. C. M. Chung, R. Dunn, H. P. Too, L. Chan.** 2000. Cloning and expression of immunoreactive antigens from *Mycobacterium tuberculosis*. *Clin. Diagn. Lab. Immunol.* **7**(4):600-606.

53. **Magnusson, L. U., A. Farewell, T. Nystrom.** 2005. ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* **13**(5):236-242.

54. **Mailaender, C., N. Reiling, H. Engelhardt, S. Bossmann, S. Ehlers, M. Niederweis.** 2004. The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Microbiology*. **150**:853-864.

55. **Manca, C., L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J. M. Musser, C. E. Barry III, V. H. Freedman, G. Kaplan.** 2001. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-α/β. *Proc. Natl. Acad. Sci. USA.* **98**(10):5752-5757.

56. **Martinez, A., S. Torello, R. Kolter.** 1999. Sliding motility in mycobacteria. *J. Bacteriol.* **181**:7331-7338.

57. **Medeiros, M. A., O. A. Dellagostin, G.R. G. Armoa, W. M. Degrave, L. de Mendonca-Lima, M. Q. Lopes, J. F. Costa, J. Mcfadden, D. McIntosh.** 2002. Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. *Microbiology*.**148**:1999-2009.

58. **Moller, A. S. W., R. Ovstebo, A. B. Westvik, G. B. Joo, K. B. F. Haug, P. Kierulf.** 2003. Effects of bacterial cell wall components (PAMPs) on the expression of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and the chemokines receptor CCR2 by purified human blood monocytes. *J. Endotoxin Res.* **9**(6):349-360.

59. **National Center for Biotechnology Information. NCBI.** [Internet]. Bethesda, MD. U.S. National Library of Medicine; 2008. Available from: http://www.ncbi.nlm.nih.gov/

60. **Nguyen, L., N. Scherr, J. Gatfield, A. Walburger, J. Pieters, C. J. Thompson.** 2007. Antigen 84, an effector of pleiomorphism in *Mycobacterium smegmatis*. *J. Bacteriol.* **189**(21):7896-7910.

61. **Niederweis, M.** 2003. Mycobacterial porins-new channel proteins in unique outer membranes. *Mol. Microbiol.* **49**(5):1167-1177.

62. **Niederweis, M.** 2008. Nutrient acquisition by mycobacteria. *Microbiology*. **154**:679-692.

63. **Onyebujoh, P., A. Zumla, I. Ribeiro, R. Rustomjee, P. Mwaba, M. Gomes, J. M. Grange.** 2005. Treatment of tuberculosis: present status and future prospects. *Bull. World Health Organ.***83**(11):857-865.

64. **Palomino, J. C., S. C. Leao, V.. Ritacco.** 2007. Tuberculosis: from basic science to patient care. Available from: http://www.tuberculosistextbook.com/

65. **Parish, T.** 2005. *Mycobacterium*: Molecular microbiology. Norfolk, U.K.: Taylor & Francis; c2005. 351 p.

66. **Post, F. A., C. Manca, O. Neyrolles, B. Ryffel, D. B. Young, G. Kaplan.** 2001. *Mycobacterium tuberculosis* 19-Kilodalton lipoprotein inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages in vitro. *Infect. Immun.* **69**(3):1433-1439.

67. **Primm, T. P., S. J. Andersen, V. Mizrahi, D. Avarock, H. Rubin, C. E. Barry III.** 2000. The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J. Bacteriol.* **182**(17):4889-4898.

68. **Raja, A.** 2004. Immunology of tuberculosis. *Indian J. Med. Res.* **120**:213-232.

69. **Raymond, J. B., S. Mahapatra, D. C. Crick, M. S. Pavelka.** 2005. Identification of the *namH* gene, encoding the hydroxylase responsible for the *N*-glycosylation of the mycobacterial peptidoglycan. **280**(1):326-333.

70. **Reichman, L. B., E. S. Hershfield.** Eds. Tuberculosis: A comprenhensive international approach. 2nd ed. New York, NY: Marcel Dekker, Inc. c2000. 898 p.

71. **Rom, W. N., S. M. Garay.** Editors. Tuberculosis. 2nd ed. Philadelphia, PA: Lippincott Williams & Wilkins; c2003. 790 p.

72. **Samanich, K. M., J. T. Belisle, M. G. Sonnenberg, M. A. Keen, S. Zolla-Pazner, S. Laal.** 1998. Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*. *J. Infect. Dis.* **178**:1534-1538.

73. **Samanich, K. M., M. A., Keen, V. D. Vissa, J. D. Harder, J. S. Spencer, J. T. Belisle, S. Zolla-Pazner, S. Laal.** 2000. *Clin. Diagn. Lab. Immunol.* **7**(4):662-668.

74. **Scott Algood, H. M., J. Chan, J. L. Flynn.** 2003. Chemokines and tuberculosis. *Cytokine Growth Factor Rev*. **14**:467-477.

75. **Singh, K. K., X. Zhang, A. S. Patibandala, P. Chien Jr., S. Laal.** 2001. Antigens of *Mycobacterium tuberculosis* expressed during preclinical tuberculosis: Serological immunodominance of proteins with repetitive amino acid sequences. *Infect. Immun.* **69**(6):4185-4191.

76. **Sorensen, A. L., S. Nagal, G. Houen, P. Andersen, A. B. Andersen.** 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect. Immun.* **63**(5):1710-1717.

77. **Stephan, J., J. Bender, F. Wolschendorf, C. Hoffmann, E. Roth, C. Mallander, H. Engelhardt, M. Niederweis.** 2005. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutriens. *Mol. Microbiol.* **58**(3):714-730.

78. **Thakur, M., P. K. Chakraborti**. 2006. GTPase activity of mycobacterial FtsZ is impaired due to its transphosphorylation by the eukaryotic-type ser/thr kinase, PknA. *J. Biol. Chem*. **281**(52):40107-40113.

79. **Triccas, J. A., T. Parish, W. J. Britton, B. Gicquel.** 1998. An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. *FEMS Microbiol. Lett*. **167**:151-156.

80. **Tullius, M. V., G. Harth, M. Horwitz.** 2001. High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather that to a protein-specific export mechanism. *Infect. Immun.* **69**(10):6348-6363.

81. **VanderVen, B. C., J. D. Harder, D. C. Crick, J. T. Belisle.** 2005. Export-mediated assembly of mycobacterial glycoproteins parallels eukaryotic pathways. *Science*. **309**:941-943.

82. **Varki, A. R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, M. E. Etzler.,** Eds. 2008. Essentials of Glycobiology. Plainview (NY): *Cold Spring Harbor Press*.

83. **World Health Organization.** 2008. Global tuberculosis control: Surveillance, planning, financing. WHO Report 2008. Available at: http://www.who.int/tb/publications/global_report/en/index.html

84. **Zuber, B., M. Chami, C. Houssin, J. Dubochet, G. Griffiths, M. Daffe.** 2008. Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *J. Bacteriol.* **190**(16):5672-5680.

CHAPTER TWO

EFFECTS OF Rel_{Mtb} ON GLUTAMINE SYNTHETASE

I. INTRODUCTION

Mycobacterium tuberculosis is a very successful intracellular pathogen. This success is due in large part to the ability of *M. tuberculosis* to manipulate the immune response of the host to the pathogen's advantage (8, 16, 25). The mechanisms for this modulation are poorly understood. However, it is known that *M. tuberculosis* is able to differentially express various proteins at specific stages of growth. Several of these proteins are able to elicit an immune response in animal models (21, 34). Therefore it is likely that these antigenic proteins are involved in the manipulation of the immune defenses of the host (25).

One such antigen is Glutamine synthetase (GlnA1, EC 6.3.1.2). GlnA1 is an oligomeric enzyme composed of 12 identical monomers, each with a molecular weight of 58 kDa. Electron microscopy data suggest that the monomers are organized in two overlying planes of 6 monomers each, an arrangement also found in the glutamine synthetases of other bacteria (1, 32). The reaction catalyzed by GlnA1 is:

$$\text{Glutamate} + NH_3 + \text{ATP} \xrightarrow[\text{GlnA1}]{} \text{Glutamine} + \text{ADP} + \text{Pi}$$

Reaction 2.1 (19)

This reaction is the only biosynthetic pathway known for the biosynthesis of glutamine in mycobacteria and other bacteria (11). The production of glutamine is

essential in nitrogen metabolism since this amino acid is utilized as a nitrogen donor in the synthesis of purines, pyrimidines and other metabolites containing nitrogen. Glutamine synthetase also plays an important role in the assimilation of ammonia (31, 33). Some metabolites that are acceptors of the amide group from glutamine are tryptophan, histidine, glucosamine-6-phosphate, and adenosine monophosphate. All of these compounds are able to inhibit the activity of glutamine synthetase by a cumulative feedback inhibition mechanism (20).

In *M. tuberculosis*, it has been reported that GlnA1 could be involved in the resistance to killing by macrophages (22, 23, 29), and is therefore considered a virulence factor (26, 28).

In this study, using protein gel electrophoresis and western blot analysis, it was found that GlnA1 was produced at a lower concentration by a mutant strain of *M. tuberculosis* (named H37Rv*Δrel*) in comparison with the wild-type strain (H37Rv). Rel is a global regulator that coordinates the stringent response, a series of changes that the bacterial cell undergoes to adapt to nutrient deprivation and other stresses (7). *In vivo* studies performed with mice have demonstrated that H37Rv*Δrel* has an impaired ability to induce granuloma formation in lungs, a hallmark of *M. tuberculosis* infection. Furthermore, mice infected with H37Rv*Δrel* were able to clear the infection and to survive without developing any of the pathological signs of *M. tuberculosis* infection (7). In the same study, microarray analysis showed that Rel influences the expression of more than 150 genes, including virulence factors, antigens, and genes involved in cell wall synthesis (7). Although that study did not show *glnA1* to be differentially regulated

by Rel_{Mtb}, our findings indicate that without Rel_{Mtb}, the production of GlnA1 is greatly reduced. Due to the role of GlnA1 in glutamine and glutamate metabolism, it has been suggested that GlnA1 is involved in regulating the pH in phagosomes containing *M. tuberculosis* cells (11). Blocking the acidification of phagosomes is a mechanism by which *M. tuberculosis* cells are able to thrive inside macrophages (27). Therefore, a lower production of GlnA1 H37Rv*Δrel* could be one of the factors that prevent this mutant from maintaining a persistent infection in mice. This potential role of GlnA1 establishing intracellular survival reinforces the initiative of studying GlnA1 as a potential drug target (3, 18, 30).

II. MATERIALS AND METHODS

Bacterial growth conditions

M. tuberculosis strains (H37Rv and H37Rv*Δrel*) were grown in 100-ml aliquots of Middlebrook 7H9 medium (Difco) supplemented with 0.05% Tween 80, 10% albumin+dextrose+catalase (ADC, Difco) and 0.2% glycerol. Cultures were incubated at 37°C with shaking for 3 weeks.

Protein preparation

50-ml aliquots of 3-week old cultures of H37Rv and H37RvΔ*rel* were centrifuged at 3,500 rpm for 5 min. Cell pellets were washed three times in phosphate buffer saline (PBS). The final pellet was resuspended in 1 ml of lysis buffer (200m M DTT, 28 mM

Tris-HCl, 22 mM Tris-Base), and added to glass beads (0.04 mm diameter) in 1.5-ml microtubes (Starstedt). Bacteria were heat-killed by placing the cell suspensions in a water bath at 70°C for 45 min, followed by cooling on ice. A protease inhibitor cocktail (Sigma) was added to the samples (50 ul/ml). Proteins were extracted using a FastPrep FP120 bead-beating device (ThermoSavant). Cells were shaken at a speed of 6.5 m/s for 45 sec and then incubated on ice for 5 min. This cycle was repeated three times. Lysed samples were centrifuged at 14,000 rpm for 10 min at 4°C to remove cellular debris. The supernatants were transferred to clean 1.5-ml plastic tubes and stored at -20°C until further use.

Protein electrophoresis

Protein electrophoresis was carried out using the Laemmli SDS-PAGE system. Protein samples were mixed with 2X SDS loading buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 25% glycerol), boiled for 10 min, and placed on ice for 5 min. The samples were loaded in a 10% polyacrylamide gel (16 x 18 cm) and electrophoresed at 200 V for 2.5 h, and then stained with Coomassie Brilliant Blue for visualization of the protein bands.

Western blot analysis

Proteins were transferred to nitrocellulose membranes using a BioRad Mini Trans-Blot cell (7.5 x 10 cm blotting area). The transfer occurred at 30 V for 3.5 h. Membranes with the transferred proteins were incubated in a 1:500 dilution of E-293

antibody (Colorado State University) in PBS + 0.5% Tween 20 + 9% skim milk for 5 h at 4°C. E-293 is a polyclonal antibody against *M. tuberculosis* whole cell lysate produced by the TB Vaccine and Research Material Contract at Colorado State University. After incubation in the primary antibody, the membranes were washed 3 times in PBS with gentle rocking. Membranes were then incubated in a 1:2500 dilution of an alkaline phospatase-labeled anti-rabbit IgG antibody (Zymed) for 4 h at room temperature and then washed 3 times in PBS. Development was carried out by incubating the membranes in alkaline phosphatase buffer + nitroblue tetrazolium chloride (NBT) + 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) for 20 min.

Protein identification

A protein band of about 85 kDa was excised from the Coomassie brilliant blue stained polyacrylamide gel. The gel band was destained for 2 h in a solution of 50% methanol + 5% glacial acetic acid in distilled water. The gel band was then dehydrated with acetonitrile, followed by reduction and alkylation with 10 mM DTT + 50 mM iodoacetamide in 100 mM NH_4HCO_3, dehydrated, rehydrated in 100 mM NH_4HCO_3, dehydrated again, and digested with 20 ng/μl trypsin in ice-cold 50 mM NH_4HCO_3. The sample was then incubated overnight at 37°C with 20 μl of 50 mM NH_4HCO_3. After overnight incubation, the solution containing the digested peptides was desalted and concentrated using C18 Zip-Tips (Millipore). The sample was analyzed by matrix assisted laser desorption/ionization using the Voyager DE RP system (Applied Biosystems). In order to identify the protein, the Mascot database (Matriz Science) was searched using monoisotopic peptide masses between the ranges 700 to 4000 Daltons that had been

detected in the sample. The 85-kDa protein was identified as glutamine synthetase from *M. tuberculosis*. This identification was confirmed by Western Blot analysis using a glutamine synthetase polyclonal antibody (anti-GlnA1) provided by Dr. Marcus Horwitz, UCLA (11).

Growth curves with different concentrations of glutamic acid and glutamine

To evaluate differences in growth between H37Rv and H37Rv*Δrel*, both strains were grown in Middlebrook 7H9 medium without glutamic acid and supplemented with 10% ADC+0.2% glycerol+ 0.05% Tween 80. Cultures began at an estimated optical density (OD_{600}) of 0.001. OD_{600} readings were taken every day for 14 days. In addition, H37Rv and H37Rv*Δrel* were grown in Middlebrook 7H9 medium without glutamic acid supplemented with 10% ADC+0.2% glycerol+ 0.05% Tween 80 and increasing concentrations of glutamine (0.2 – 20 mM). These cultures were also started at an estimated initial optical density (OD_{600}) of 0.001, and OD_{600} readings were taken every day for 14 days.

III. RESULTS

a. Identification of GlnA1

When protein lysates of H37Rv and H37Rv*Δrel* were compared side by side using SDS-PAGE, various protein bands appeared to be differentially expressed between these two strains. One of the bands seemed to be present in a much higher concentration in the

H37Rv lysate than in the H37Rv*Δrel* lysate. This band was identified by mass-spectroscopy as *M. tuberculosis* glutamine synthetase (GlnA1, EC 6.3.1.2)(Figure 2.1).

Despite the high level of confidence given by the MASCOT database for the identification of the protein, the estimated molecular weight of the excised band (~85 kDa) did not correspond to the reported molecular weight for GlnA1 (58 kDa). Therefore, Western blot analysis using a polyclonal antibody specific for GlnA1 was used to help verify this result. The antibody, kindly provided by Dr. Harth and Dr. Horwitz at UCLA, reacts very strongly to GlnA1 even at a dilution of 1:10,000. This sensitivity makes it excellent for evaluating the levels of expression of GlnA1 by comparing the signal from different concentrations of protein. Indeed, by using the anti-GlnA1 antibody two goals were accomplished: first, the identity of the protein band was confirmed as GlnA1; and second, by loading two different amounts of the protein lysates, the preliminary observation in which more of GlnA1 was detected in the H37Rv sample in comparison with the H37Rv*Δrel* sample, was also confirmed (Figure 2.2).

The difference in the reported molecular weight of GlnA1 and the estimated molecular weight of our band was attributed to the pre-stained protein ladder used (Invitrogen). Pre-stained ladders are used to obtain a broad assessment of the molecular weight of the proteins, and can be misleading when used to determine specific masses.

b. GlnA1 is expressed at higher levels in H37Rv than in H37Rv*Δrel*

Western blot analysis was used to compare the expression levels of GlnA1 in H37Rv and H37Rv*Δrel*. A polyclonal antibody reactive to *M. tuberculosis* whole-cell lysate (E-293 TBVTRM/Colorado State University) was used and the levels of GlnA1 expressed by H37Rv were found to be approximately 4-fold higher than those of H37Rv*Δrel* (Figure. 2.3). This difference in expression was confirmed by the use of an anti-GlnA1 antibody (Figure. 2.2). Figure 2.2 also shows that the difference in the Western blot signal for GlnA1 between H37Rv and H37Rv*Δrel* varies in proportion to the concentration of protein present in the membrane and this difference remains constant when using different amounts of sample.

c. In the absence of glutamate, H37Rv*Δrel* shows a delayed start of the logarithmic phase in comparison with H37Rv.

The substrate for the synthesis of glutamine by GlnA1 is glutamate. If glutamate is in short supply or absent, GlnA1 production is inhibited until glutamate is available (4). In the absence of glutamate the cell has to synthesize this amino acid through the reaction catalyzed by glutamate dehydrogenase and glutamate synthase. Once glutamate has been synthesized, it can be used by GlnA1 to produce glutamine, which in turn is utilized as a nitrogen donor for the synthesis of other molecules (5). The goal of this experiment was to explore the possibility that Rel_{Mtb} would affect other reactions of the metabolism of nitrogen, besides those catalyzed by GlnA1, causing growth defects. Indeed, when *M. tuberculosis* strains were grown in the absence of glutamate, it was

seen that H37Rv*Δrel* has a prolonged lag phase, entering logarithmic growth significantly later than H37Rv and not attaining the same growth density of the wild-type strain in the period evaluated (Figure 2.4).

d. In the absence of glutamate, the addition of glutamine to the medium does not improve significantly the growth of H37Rv*Δrel*

We explored the effect of supplementing the medium with different concentrations of glutamine, in the absence of glutamate. The hypothesis here was that H37Rv*Δrel* would be able to compensate for its lower GlnA1 production and resulting lower synthesis of glutamine by utilizing external glutamine provided in the medium. The concentrations of glutamine added to the medium were 0.2 mM, 1.0 mM, 2.0 mM and 20.0 mM. For H37Rv*Δrel*, we were expecting to see growth equivalent to that of H37Rv at some of the concentrations of glutamine.

For H37Rv the addition of 1.0 mM of glutamine slightly improved the growth of the cells in comparison with their growth when no glutamine was present. Adding more than 1.0 mM of glutamine to the medium did not improve the cell growth, in fact, the addition of 20.0 mM seemed to have an inhibitory effect (Figure 2.5).

The results for H37Rv*Δrel* are more uniform (Figure 2.6). All concentrations seemed to enhance to some extent the growth of this strain, although none of the concentrations appeared to have a dramatic effect on growth.

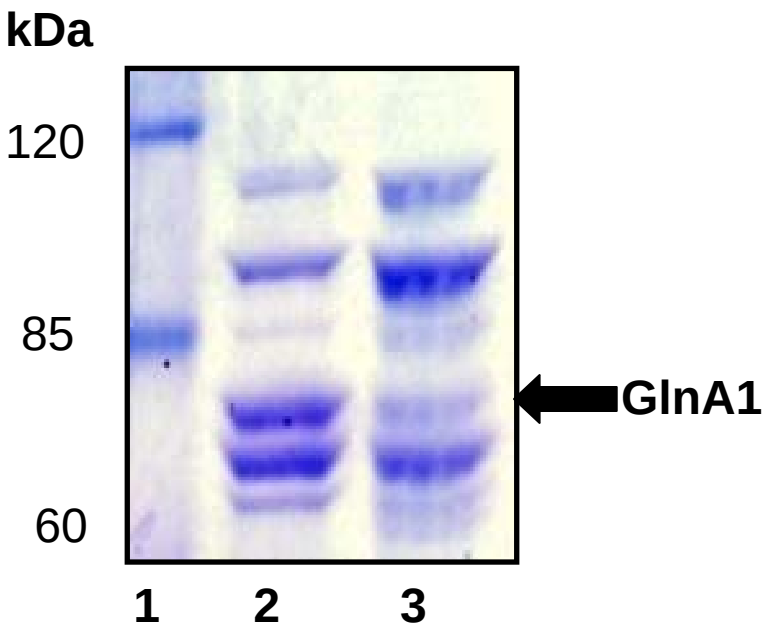

Figure 2.1. **SDS-PAGE comparison of protein lysates of H37Rv and H37Rv*Δrel*.** Proteins were run in a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1. Pre-stained protein ladder. Lane 2. H37Rv protein whole cell lysate. Lane 3. H37Rv*Δrel* protein whole cell lysate.

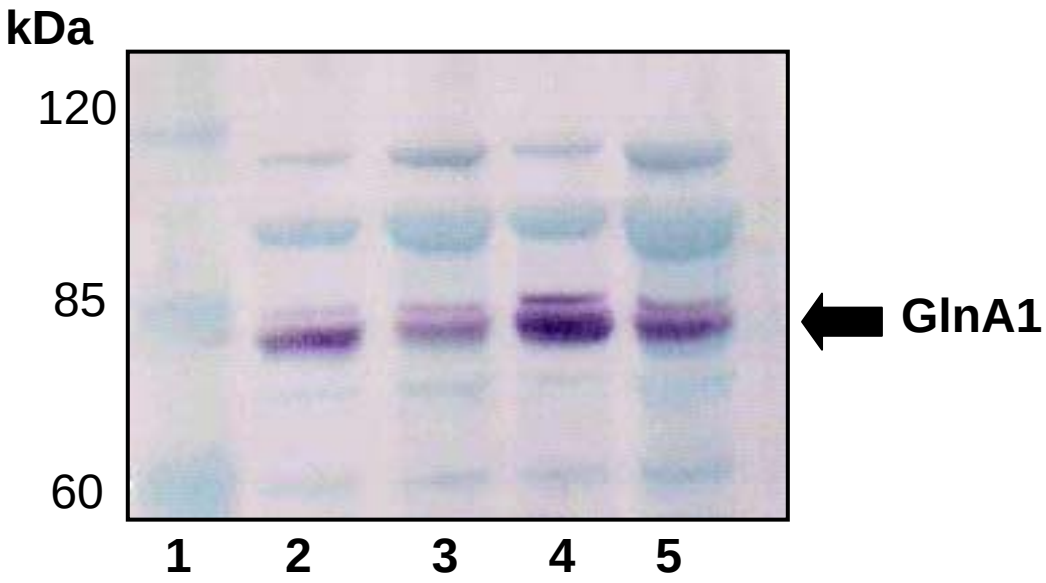

Figure 2.2. **Confirmation of GlnA1 identification by Western-blot analysis**. Proteins were run in a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue. The gel was de-stained in methanol and acetic acid and transferred to a nitrocellulose membrane. An anti-GlnA1 antibody was used to detect GlnA. Lane 1. Pre-stained protein ladder. Lane 2. H37Rv protein whole cell lysate. Lane 3. H37Rv*Δrel* protein whole cell lysate. Lane 4. H37Rv protein whole cell lysate. Lane 5. H37Rv*Δrel* protein whole cell lysate.

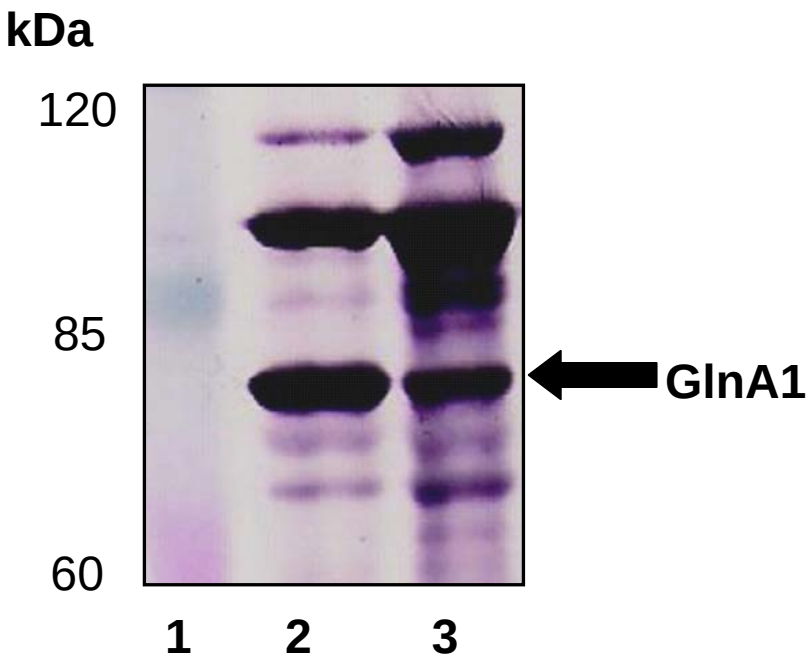

Figure 2.3.Western blot Comparison of GlnA1 expression levels between H37Rv and H37Rv*Δrel*. Proteins were run in a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. An anti-*M. tuberculosis* whole cell lysate polyclonal antibody was used as the primary antibody. Lane 1. Pre-stained protein ladder. Lane 2. H37Rv protein whole cell lysate. Lane 3. H37Rv*Δrel* protein whole cell lysate.

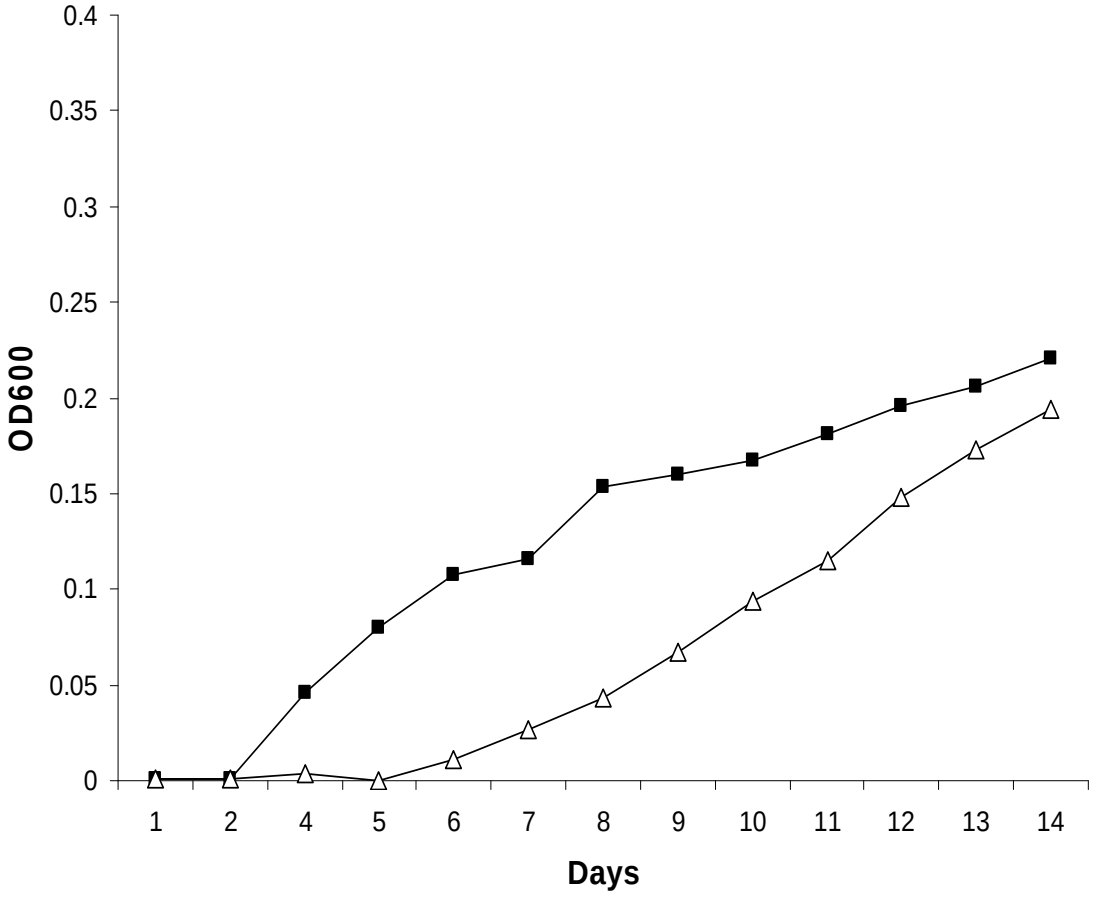

Figure 2.4. Growth comparison of H37Rv and H37RvΔ*rel* in the absence of glutamic acid. Strains were grown on Middlebrook 7H9 medium+ADC+0.2%glycerol+0.05%Tween 80 without glutamic acid. Optical densities (OD) at 600 nm were taken daily over a period of 14 days. ■ H37Rv △ H37Rv*Δrel*

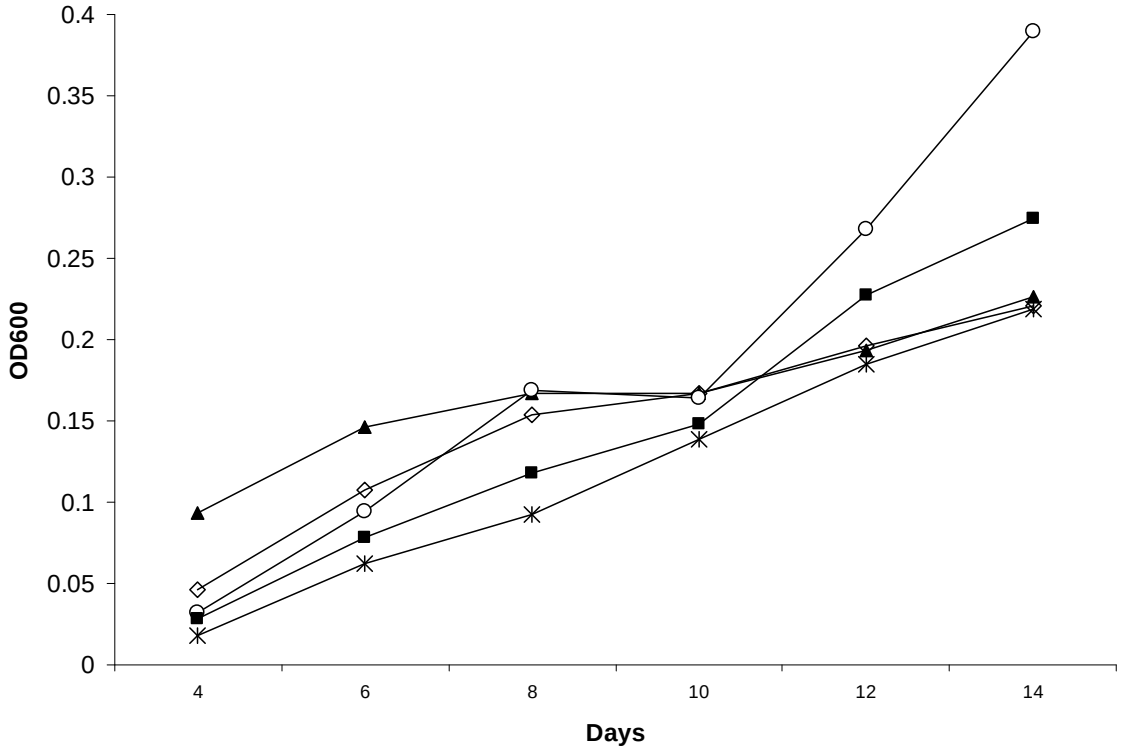

Figure 2.5. Growth of H37Rv in the absence of glutamic acid and different concentrations of glutamine. Strains were grown on Middlebrook 7H9 medium + ADC + 0.2% glycerol + 0.05%Tween 80 without glutamic acid, with increasing concentrations of glutamine (0–20mM) Optical densities (OD) at 600 nm were taken daily over a period of 14 days. ◇ 0.0 mM, ▲ 0.2 mM, ○ 1.0 mM, ■ 2.0 mM, ∗ 20.0 mM.

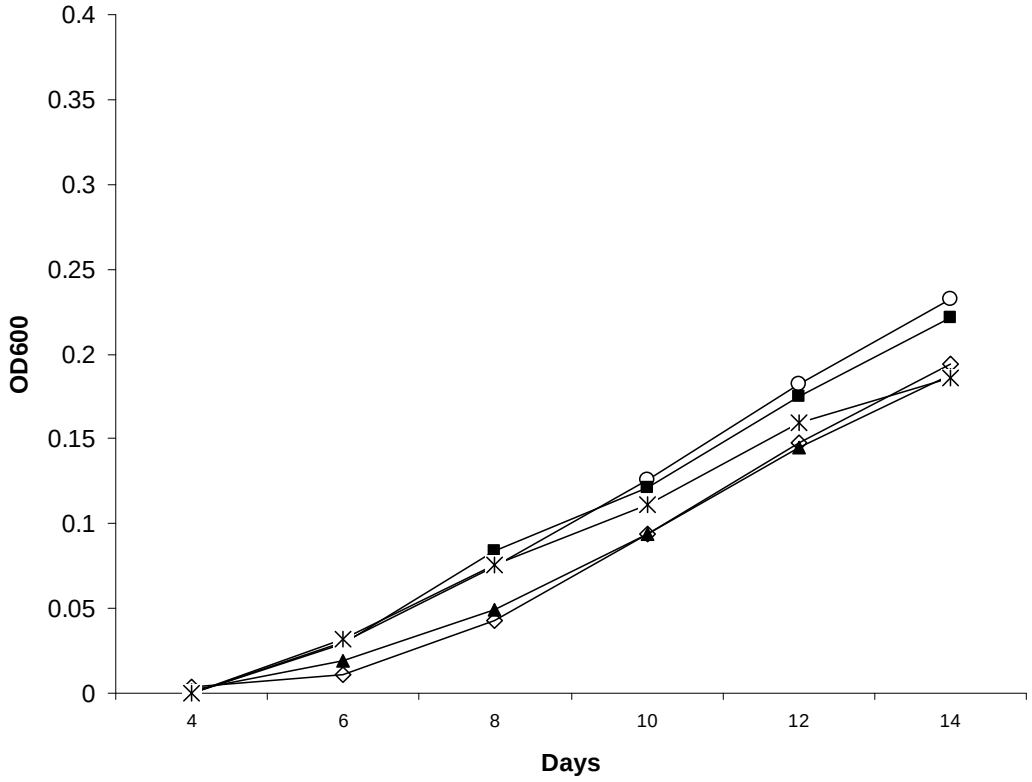

Figure 2.6. Growth of H37Rv*Δrel* in the absence of glutamic acid and different concentrations of glutamine. Strains were grown on Middlebrook 7H9 medium + ADC + 0.2% glycerol + 0.05%Tween 80 without glutamic acid, with increasing concentrations of glutamine (0–20mM) Optical densities (OD) at 600 nm were taken daily over a period of 14 days. ◇ 0.0 mM, ▲ 0.2 mM, ○ 1.0 mM, ■ 2.0 mM, ∗ 20.0 mM.

IV. DISCUSSION

Previous studies performed on Glutamine synthetase (GlnA1) of *M. tuberculosis* have focused on its antigenic nature and its essential role in *M. tuberculosis* metabolism (11-15). This however, is the first report that associates GlnA1 with Rel, the global regulator that coordinates the stringent response in mycobacteria. The stringent response comprises the metabolic changes that the bacterial cell undergoes to adapt to nutrient deprivation, other environmental stresses, and possibly to survive within the host.

In this study, we analyzed the protein profiles of H37Rv*Δrel* (an *M. tuberculosis* strain lacking rel_{Mtb}) and H37Rv (*M. tuberculosis* wild-type) by using SDS-PAGE and Western-Blot analyses (Figures 2.1, 2.2). Both approaches showed that there is clearly more GlnA1 expressed in H37Rv than in H37RvΔ*rel* (Figures 2.1 – 2.3). This difference is related to the absence of Rel in H37Rv*Δrel*, as evidenced by finding the same levels of GlnA1 in a Rel_{Mtb} complemented strain (data not shown). This differential expression of GlnA1 between these two strains indicates that Rel plays a role in the regulation of GlnA1 and further supports the hypothesis that Rel influences the expression of antigens and genes involved in metabolism.

In a previous study in which H37Rv*Δrel* was exposed to nutrient deprivation, it was found by microarray analysis that more than 150 genes were under the regulation of Rel_{Mtb}. Among these were genes involved in cell wall formation, virulence factors, and antigens (7). The report did not list *glnA1* as one of the genes regulated by Rel_{Mtb}. The

discrepancy between results could be due to differences in the growth conditions used in each study; for the microarray analysis cells were grown in a nutritionally-deficient medium, consisting only of Tris-buffered saline and Tween (TBST) for no more than 6 hours (7), while for the present study, cells were grown in nutrient-rich Middlebrook 7H9 medium for 3 weeks. It is also important to note that the levels of messenger RNA in microarray analysis may not always reflect the levels of protein produced. In studies comparing protein and messenger RNA levels it was found that for several genes that appear unchanged in the microarray data, the protein levels actually varied by up to 20-fold (9, 10).

It has been reported that at least one-third of the total GlnA1 produced in the cell is exported to the extracellular environment (12, 30). This exported GlnA1 could help manipulate the pH of phagosomes containing *M. tuberculosis* cells, preventing acidification and allowing *M. tuberculosis* cells to survive and multiply inside these phagosomes (12, 27). If that is indeed the case, then reduced expression of GlnA1 in H37RvΔ*rel* could in part be responsible for the inability of this strain to maintain a persistent infection in mice, as it has been reported (7). To test this hypothesis, it would be necessary to evaluate the pH of phagosomes containing dead and live *M. tuberculosis* cells. In previous studies it has been shown that acidified phagosomes contain only dead mycobacteria, while those containing live mycobacteria have a more neutral pH (2, 6). It will be also important to measure the amount of GlnA1 in these same phagosomes in order to establish if there is a correlation between levels of GlnA1 and phagosome acidification. We would expect to find a lower pH inside phagosomes containing H37Rv*Δrel* cells than in those containing H37Rv cells.

Relevant to this study are three enzymes involved in nitrogen metabolism in mycobacteria: GlnA1, glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT)(5).

When *M. tuberculosis* is grown in media containing glutamate and ammonium, GlnA1 is activated and it catalyzes the synthesis of glutamine (reaction 2.1). In contrast, in the absence of glutamate, GlnA1 is inhibited, but GDH and GOGAT are activated to synthesize glutamate from oxoglutarate (5). The newly synthesized glutamate can then be used to produce glutamine. The availability of glutamine is essential for the growth of *M. tuberculosis* cells since this amino acid is used as nitrogen donor in the synthesis of several metabolites (13, 14).

We decided to study the growth of H37Rv and H37Rv*Δrel* in the absence of glutamate because of the central role this amino acid plays in mycobacterial metabolism. When both strains were grown in liquid media without glutamate, we found that H37Rv*Δrel* took longer than H37Rv to begin the logarithmic phase (Figure 2.4), likely because of the lower amount of GlnA1 produced by H37Rv*Δrel.* From our experiments it seems that both strains are able to cope with the lack of glutamate, indicating that both strains can synthesize glutamate from oxoglutarate. However, the slower growth of H37Rv*Δrel* suggests that once glutamate has been synthesized, there is not enough GlnA1 to catalyze the production of glutamine, which can have a deleterious effect on the cells metabolism and may ultimately translate into impaired growth.

The addition of glutamine to the media without glutamate did not have a significant effect in any of the strains. It appears that in the case of H37Rv, the addition of 1.0 mM of glutamine (and to a lesser extent of 2.0 mM) slightly improved the growth of the cells (Figure 2.5). However, we observed that adding higher concentrations of this amino acid to the media inhibited the growth of H37Rv. For H37Rv*Δrel*, none of the different concentrations of glutamine appeared to improve the growth of the cells (Figure 2.6). Furthermore, this strain was not able to attain the growth rate of H37Rv regardless of amount of glutamine added. The lack of response to the addition of glutamine to the media is probably related to the low ability of *M. tuberculosis* to transport this amino acid from its environment to the cytoplasm (31).

It is important to highlight that the growth of both strains in all conditions tested was significantly slower than when the cells are grown in regular Middlebrook 7H9 medium containing glutamate (data not shown). An explanation for this could be the extremely low inoculum size used to start the cultures, the lightness of which was necessary to reduce the amount of glutamate or glutamine carried over from the parental culture (31).

To obtain a better understanding of the effect of glutamate and glutamine on H37Rv and H37Rv*Δrel*, the growth curves reported here should be repeated but the duration of the experiments should be increased at least to the point where both strains reach stationary phase. Given that the initial inoculum needs to be extremely small, it would probably take 4 to 5 weeks to attain this phase.

Another role that has been proposed for GlnA1 in *M. tuberculosis* is the synthesis of poly-L-glutamic acid/glutamine (P-L-glx), a cell wall structure found in pathogenic but not in non-pathogenic mycobacteria (15, 24). It would be interesting to compare the levels of P-L-glx between H37Rv and H37Rv*Δrel*, because lower levels of this compound in the latter could help explain its inability to sustain an infection in mice (7).

To our knowledge, this is the first time that Rel_{Mtb} is shown to affect the production of GlnA1. Our results offer a starting point to continue working with these two proteins. The demonstration of a link between GlnA1 and the inability of H37Rv*Δrel* to survive for long periods of time inside infected macrophages and to maintain a persistent infection in mice, will support the hypothesis that Rel_{Mtb} is involved not only in the regulation of the stringent response, but that the expression of virulence factors that play an essential role in the pathogenesis of TB also depends on this protein.

V. REFERENCES

1. **Almassy, R. J, C. A. Janson, R. Hamlin, N-H, Xoung, D. Eisenberg.** 1986. Novel subunit- subunit interactions in the structure of glutamine synthetase. *Nature* ***323***: 304-309

2. **Armstrong, J.A., P. D'Arcy Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* **134**(3):713-739.

3. **Berlicki, L., A. Obojska, G. Forlani, P. Kafarski.** 2005. Design, synthesis, and activity of analogues of phosphinothricin as inhibitors of glutamine synthetase. *J. Med. Chem.* **48**:6340-6349.

4. **Brown, J. R., Y. Masuchi, F.T. Robb, W. F. Doolittle.** 1994. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. *J. Mol. Evol.* **38**:566-576.

5. **Burkovski, A.** 2003. Ammonium assimilation and nitrogen control in *Corynebacterium glutamicum* and its relatives: an example for new regulatory mechanisms in actinomycetes. **27**:617-628.

6. **Crowle, A. J., R. Dahl. E.Ross, M. H. May.** 1991. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* and *Mycobacterium avium* in cultured macrophages are not acidic. *Infect. Immun.* **59**(5):1823-1831.

7. **Dahl, J. L., C. N. Kraus, H. I. M. Boshoff, B. Doan, K. Foley, D. Avarock, G. Kaplan, V. Mizrahi, H. Rubin, C. E. Barry III.** 2003. The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc. Natl. Acad. Sci. USA.* **100**:10026-10031.

8. **Gehring A. J., K. M. Dobos, J. T. Belisle, C. V. Harding, W. H. Boom.** 2004. *Mycobacterium tuberculosis* LprG (*Rv1411c*): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J. Immunol.* **173**:2660-2668.

9. **Griffin, T. J., S. P. Gygis, T. Ideker, B. Rist, J. Eng, L. Hood, R. Aebersold.** 2002. Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Mol. Cell. Prot.* **1**:323-333.

10. **Gygi, S. P., Y. Rochon, B. R. Franza, R. Aebersold.** 1999. Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**(3):1720-1730.

11. **Harth, G., D. L. Clemens, M. A. Horwitz.** 1994**. Glutamine synthetase of *Mycobacterium tuberculosis***: Extracellular release and characterization of its enzymatic activity. *Proc. Natl. Acad. Sci. USA.* **91**:9342-9346.

12. **Harth, G., M. A. Horwitz.** 1997. Expression and efficient export of enzymatically active *Mycobacterium tuberculosis* glutamine synthetase in *Mycobacterium smegmatis* and evidence that the information for export is contained within the protein. *J. Biol. Chem*. **272**(36):22728-22735.

13. **Harth, G., M. A. Horwitz.** 1999. An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. *J. Exp. Med*. **189**(9):1425-1435.

14. **Harth, G., S. Maslesa-Galic, M. V. Tullius, M. A. Horwitz.** 2005. All four *Mycobacterium tuberculosis glnA* genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis. *Mol. Microbiol.* **58**(4):1157-1172.

15. **Harth, G., P. C. Zamecnik, J. Y. Tang, D. Tabatadze, M. A. Horwitz.** 2000. Treatment of Mycobacterium tuberculosis with antisense oligonucleotides to glutamine synthetase mRNA inhibits glutamine synthetase activity, formation of the poly-L-glutamate/glutamine cell wall structure, and bacterial replication *Proc. Natl. Acad. Sci. USA*. **97**(1):418-423.

16. **Korf, J., A. Stoltz, J. Verschoor, P. De Baetselier, J. Grooten.**2005. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *Eur. J. Immunol.* **35**:890-900.

17. **Krajewski, W. W., T. A. Jones, S. L. Mowbray.** 2005. Structure of *Mycobacterium tuberculosis* glutamine synthetase in complex with transition-state mimic provides functional insights. *Proc. Natl. Acad. Sci. USA.* **102**(30):10499-10504.

18. **Krajewski, W. W.** 2008. Structural studies of glutamine synthetases: Towards the development of novel antitubercular agents. Uppsala, Sweeden. *Acta Universitatis Upsaliensis. Digital Comprenhensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology*. **553.** 48p.

19. **Kustu, S. G., K. McKereghan**. 1975. Mutations affecting glutamine synthetase activity in *Sallmonella typhimurium*. *J. Bacteriol.* **122**(3):1006-1016.

20. **Liaw, S. H., C. Pan, D. Eisenberg.** 1993. Feedback inhibition of fully unadenylylated glutamine synthetase from *Salmonella typhimurium* by glycine, alanine, and serine. *Proc. Natl. Acad. Sci. USA.* **90**:4996-5000.

21. **Mendelson, M., S. Walters, I. Smith, G. Kaplan.** 2005. Strain-specific mycobacterial lipids and the stimulation of protective immunity to tuberculosis. *Tuberculosis* **85**:407-413.

22. **Miller, B. H., T. M. Shinnick.** 2000. Evaluation of *Mycobacterium tuberculosis* genes involved in resistance to killing by human macrophages. *Infect. Immun.* **68**(1):387-390.

23. **Miller, B. H., T. M. Shinnick.** 2001. Identification of two *Mycobacterium tuberculosis* H37Rv ORFs involved in resistance to killing by human macrophages. *BMC Microbiol.* **1**:26 Available at: http://www.biomedcentral.com/1471-2180/1/26

24. **Phiet, P. H., J. Wietzerbin, E. Zissman, J. F. Petit, E. Lederer.** 1976. Analysis of the cell wall of five strains of *Mycobacterium tuberculosis* BCG and of an attenuated human strain, W115. *Infect. Immun.* **13**(3):677-681.

25. **Rao, V., N. Dhar, A. K. Tyagi.** 2003. Modulation of host immune responses by overexpression of immunodominant antigens of *Mycobacterium tuberculosis* in Bacille Calmette-Guerin. *Scand. J. Immunol.* **58**:449-461.

26. **Raynaud, C., G. Etienne, P. Peyron, M. A. Laneelle, M. Daffe.** 1998. Extracellular enxymes activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis. Microbiology*. **144**:577-587.

27. **Rhoades, E. R., H. J. Ullrich.** 2000. How to establish a lasting relationship with your host: Lessons learned from *Mycobacterium* spp. *Immunol. Cell Biol.* **78**:301-310.

28. **Smith, I.** 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin. Microbiol. Rev.* **16**(3):463-496.

29. **Triccas, J. A., B. Gicquel.** 2000. Life on the inside: Probing *Mycobacterium tuberculosis* gene expression during infection. *Immunol. Cell Biol.* **78**:311-317

30. **Tullius, M. V., G. Harth, M. Horwitz.** 2001. High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than to a protein-specific export mechanism. *Infect. Immun.* **69**(10):6348-6363.

31. **Tullius, M. V., G. Harth, M. Horwitz.** 2003. Glutamine synthetase GlnA1 is essential for growth of *Mycobacterium tuberculosis* in Human THP-1 macrophages and Guinea pigs. *Infect. Immun.* **71**(7):3927-3936.

32. **Valentine R. C., B. M. Shapiro, E. R. Stadtman.** 1968. Regulation of Glutamine Synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry*. **7**(6):2143-2152.

33. **Wayne, L. G., C. D. Sohaskey.** 2001. Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu. Rev. Microbiol.* **55**:139-163.

34. **Yeremeev, V.V., I. V. Lyadova, B. V. Nikonenko, A. S. Apt, C. Abou-Zeid, J. Inwald, D. B. Young.** 2000. The 19-kD antigen and protective immunity in a murine model of tuberculosis. *Clin Exp Immunol.* **120**:274-279.

CHAPTER 3

EFFECT OF Rel_{Mtb} on $Wag31_{Mtb}$

I. INTRODUCTION

Despite being one of the first infectious diseases recognized in humans, tuberculosis (TB) is still a major public health problem around the World. One of the reasons for the inability to eradicate this disease is the capacity of the causative agent, *Mycobacterium tuberculosis*, to evade the immune system of infected individuals by remaining in a dormant state for years and even decades inside lung granulomas. *M. tuberculosis* cells are able to awake from this dormancy and resume normal metabolic activity and full infectious capacity. This reactivation often accompanies an impairment of the host immune response, such as what occurs due to infection with HIV, treatment with immunosuppressive drugs, advanced age, malnutrition, stress, and alcoholism (25). This ability to switch between a dormant state and a metabolically active replicating state is not fully understood. However, it is clear that in order to accomplish this, *M. tuberculosis* must be able to efficiently regulate all components of the cell division process (3, 17).

As in other bacteria, cell division in *M. tuberculosis* involves the segregation of newly-replicated DNA, synthesis of cell wall components, and the distribution of cytoplasmic material between daughter cells (Hett 2008). One of the major proteins involved in cell division is $FtsZ_{MTB}$ (4, 11). FtsZ is a tubulin-like protein that assembles at mid-cell during the cell division process, where it forms a ring around the cell just under the plasma membrane. This structure is further developed into what is known as the Z ring (11, 17).

Once FtsZ has localized at the division site, a series of proteins are recruited, these include proteins involved in the segregation process, synthesis of peptidoglycan and other cell wall components (11). Meanwhile, the chromosomal DNA undergoes replication and is further divided into the future daughter cells (17). Once the chromosomal DNA and the cytoplasmic components have been segregated, a peptidoglycan septum is formed at the division site. Subsequently, peptidoglycan degrading enzymes arrive at the division site where they degrade septal peptidoglycan and allow for separation of daughter cells (17). The proper localization of FtsZ at the beginning of the division process is crucial to generate normal daughter cells, and is therefore closely regulated. In *E. coli* the localization of FtsZ and subsequent formation of the Z ring are controlled by MinC, MinD and MinE proteins that together form the Min system (19). MinC inhibits the assembly of FtsZ; MinD binds MinC and anchors it to the cell poles; and MinE maintains MinCD localized to the poles (17). The system guarantees that the cell division occurs in the middle of the cell and away from the poles. *M. tuberculosis* and mycobacteria in general, lack MinC or MinD proteins, however they do have an orthologue of MinE, known as DivIVA (16, 30).

In *M. tuberculosis*, DivIVA is the product of the $wag31_{Mtb}$ gene and it is also known as both Wag31 and Antigen 84 (34). $wag31_{Mtb}$ has been reported as an essential gene in *M. tuberculosis* (20, 21, 30). The protein which hereafter will be referred to as $Wag31_{Mtb}$, is a 34 kDa protein and is highly conserved in mycobacteria (16). It was originally identified as an antigen in *M. bovis*, and later in *M. tuberculosis* (6). It has been reported as the substrate of PknB, a protein kinase also involved in the process of cell division (20, 21).

In our lab, we found that $Wag31_{Mtb}$ was expressed at higher levels in wild-type *M. tuberculosis* (H37Rv) compared with the expression levels in a strain deficient for the stringent response (H37RvΔrel_{Mtb}). Rel_{Mtb} is a global regulator that coordinates the stringent response in *M. tuberculosis*. This response comprises all the metabolic changes that cells undergo in order to adapt to nutrient deprivation, and other environmental stresses, such as those encountered inside the host (9). Rel_{Mtb} is thought to be crucial in the adaptation of *M. tuberculosis* to the granuloma environment, a key feature in tuberculosis pathogenesis. It is inside lung granulomas that *M. tuberculosis* remains in a dormant state for years (25). The strain H37RvΔrel_{Mtb} is unable to maintain a persistent infection in mice and it has a greatly reduced ability to induce granuloma formation (9). Given the essential role that $Wag31_{Mtb}$ plays in cell division, our finding that H37RvΔrel_{Mtb} has a lower expression of this protein, could help explain the defects reported for H37RvΔ rel_{Mtb}.

In this work we describe our findings regarding the differential expression of Wag31 between H37Rv and H37RvΔrel_{Mtb}. Several experiments that aimed to analyze the effects of $Wag31_{Mtb}$ in the absence of Rel were performed in *M. smegmatis.* We decided to use this species mainly because it is not pathogenic and it grows significantly faster than *M. tuberculosis*. These characteristics make *M. smegmatis* an excellent surrogate system to study *M. tuberculosis* genes. We transformed a wild-type strain of *M. smegmatis* (mc^2155) and a *rel* knock-out strain ($mc^2155\Delta rel$) with $Wag31_{Mtb}$. We evaluated these two strains in terms of differences in colony morphology, lipid profiles, cytokine production by infected macrophages, and intracellular survival.

II. MATERIALS AND METHODS

Strains and culture conditions

M. tuberculosis strains (H37Rv and H37Rv*Δrel*) were grown in Middlebrook 7H9 medium supplemented with albumin, dextrose and catalase (ADC, Difco) and 0.2% glycerol + 0.05% Tween 80. Cultures were grown to stationary phase at 37°C in a shaking incubator. *M. smegmatis* strains (mc^2155, mc^2155*Δrel*) were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol + 0.05% Tween 80 at 37°C with shaking. *M. smegmatis* containing the $wag3_{Mtb}$ gene on a multicopy plasmid (pOLYG)(mc^2155/p$wag31_{Mtb}$ and mc^2155*Δrel*/p$wag31_{Mtb}$) were grown as described above, with the addition of hygromycin (50 μl/ml) to the culture medium. In order to compare the colony morphology of the four *M. smegmatis* strains, they were inoculated on Middlebrook 7H10 agar, and incubated for 5 days at 37°C.

Protein preparation for rabbit immunization

Fifty-ml aliquots of 3-week old cultures of H37Rv and H37Rv*Δrel* were centrifuged at 3,500 rpm for 5 min and the cell pellets were washed three times in phosphate buffer saline (PBS). Final cell pellets were resuspended in 1 ml of lysis buffer (200 mM DTT, 28 mM Tris-HCl, 22 mM Tris-Base), and added to glass beads (0.04 mm diameter) in 1.5 ml microtubes (Starstedt). Bacteria were heat-killed at 70°C in a water bath for 45 min, followed by immediate cooling on ice. A protease inhibitor cocktail (Sigma) was added to the samples to a final concentration of 50 μl/ml. Proteins were extracted using a

FastPrep FP120 bead-beating device (ThermoSavant). Cells were shaken at a speed of 6.5 m/s for 45 sec and then incubated on ice for 5 min. This cycle of cell lysis was repeated five times before the samples were boiled for ten minutes to enhance cell lysis. Following boiling, the samples were bead-beaten again five more times. Lysed samples were centrifuged at 14,000 rpm for 10 min at 4°C to remove unlysed cells and cellular debris. Lysate supernatants were filter sterilized and stored at -20°C until further use.

Polyclonal antibody production

White New Zealand female rabbits were immunized subcutaneously with an emulsion of protein whole cell lysate from H37Rv or H37RvΔ*rel* and Titermax Gold adjuvant (Sigma), prepared according to the manufacturer instructions. The rabbits were immunized three times over a period of six weeks. Before each immunization, marginal ear bleedings were performed to evaluate the reactivity of the antisera against the *M. tuberculosis* proteins by Western blot analysis. Two weeks after the final immunization, approximately 75 ml of blood were obtained from each rabbit by cardiac terminal bleed. The blood was allowed to coagulate and the sera were separated from the clots. The serum obtained from each rabbit was aliquoted and stored at -80°C.

Protein electrophoresis

Protein electrophoresis was carried out according to the Laemmli SDS-PAGE system. Each protein sample was mixed with 2X SDS loading buffer, boiled for 10 min, and placed on ice for 5 min. The samples were loaded in a 10% polyacrylamide gel (16 x 18

cm). A protein standard was also loaded on the gel. Proteins were electrophoresed at 200 V for 2.5 h, and then stained with Coomassie Brilliant Blue for visualization of the protein bands.

Western Blot

Proteins were transferred to nitrocellulose membranes using a BioRad Mini Trans-Blot cell (7.5 x 10 cm blotting area), as previously described (8). The transfer occurred at 30 V for 3.5 h. A 1:3000 dilution of H37Rv-immunized rabbit serum, prepared in PBS + 0.5% Tween 20 + 9% skim milk, was pre-absorbed with 1 ml of H37Rv*Δrel* whole cell lysate and incubated at 4°C overnight with rocking. The rabbit serum treated in this manner was used as the primary antibody in subsequent Western blots. The membranes with the transferred proteins were incubated in the primary antibody 5 h at 4°C. After this incubation, the membranes were washed three times in PBS with gentle rocking. Membranes were then incubated in a 1:2500 dilution of an alkaline phospatase labeled anti-rabbit IgG antibody (Zymed) for 4 h at room temperature before washing three times in PBS. Development was carried out by incubating the membranes in alkaline phospatase buffer (formula) + nitroblue tetrazolium chloride (NBT) + 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) for 20 min.

Protein identification

A protein band of about 40-kDa was excised from the polyacrylamide gel stained with Coomassie Brilliant blue. The gel band was destained for 2 h in a solution of 50% methanol + 5% glacial acetic acid in distilled water. The gel band was dehydrated with acetonitrile, followed by reduction and alkylation with 10 mM DTT + 50 mM iodoacetamide in 100 mM NH_4HCO_3, dehydrated, rehydrated in 100 mM NH_4HCO_3, dehydrated again, and digested with 20 ng/μl trypsin in ice-cold 50 mM NH_4HCO_3. The sample was incubated overnight at 37°C with 20 μl of 50 mM NH_4HCO_3. After this incubation, the solution containing the digested peptides was desalted and concentrated using C18 Zip-Tips (Millipore). The sample was analyzed by matrix assisted laser desorption/ionization using the Voyager DE RP system (Applied Biosystems). In order to identify the protein, the MASCOT database (Matrix Science) was searched for monoisotopic peptide masses between the ranges 700 to 4000 Daltons detected in the sample. The 40-kDa protein was identified as the *M. tuberculosis Rv2145* gene product, also known as $Wag31_{Mtb}$.

Cloning of *wag31* into *M. smegmatis*

The *wag31* gene, including a 350-bp upstream region, was amplified from *M. tuberculosis* genomic DNA using the primers 5'-CTGGTTGCGTTCATCGGTAT-3' and 5'-GAAAACTGGCGCGTGTCC-3'. The resulting PCR product was cloned into pDrive cloning vector (Qiagen). After digestion with ApaI and PstI, the DNA fragment was cloned into the pOLYG shuttle vector (14) and the resulting plasmid was named p*wag31*.

This construct was electroporated into *M. smegmatis* cells to produce the strains mc^{2}155/p*wag31*$_{Mtb}$ and mc^{2}155*Δrel*/p*wag31*$_{Mtb}$.

Isolation of mRNA and reverse transcriptase-PCR

mRNA from all *M. smegmatis* strains was obtained by using TRIzol (Invitrogen), following the manufacturer directions. Briefly, mycobacterial cells grown to stationary phase were harvested by centrifugation. The resulting pellets were resuspended in TRIzol, transferred to 1.5 ml screw-cap plastic tubes, and mixed with glass beads (0.4 mm diameter). The tubes were shaken using a FastPrep FP120 bead-beating device (ThermoSavant). Cells were shaken at a speed of 6.5 m/s for 45 sec before incubating on ice for 5 min. This cycle was repeated three more times. The samples were then centrifuged at 12,000 x *g* and 4ºC for 10 min, to remove cell debris and glass beads. The supernatant was transferred to a 1.5-ml RNAse-free microfuge tube containing chloroform:isoamyl alcohol (24:1). After 2 minutes of continuous shaking, the samples were centrifuged at 12,000 x *g* for 10 minutes. The upper aqueous layer was transferred to a tube containing isopropanol and incubated at 4ºC overnight. Tubes were centrifuged at 13,000 x *g* for 20 min to harvest the nucleic acid. The resulting pellets were treated with DNAse I (Roche) for 15 min at room temperature. Following DNAse treatment, the mRNA was cleaned up using the RNeasy kit (Qiagen). cDNA from each strain was generated using Superscript III Reverse Transcriptase (Invitrogen) following manufacturer instructions. The resulting cDNA was used as template to amplify the gene *Rv2145* (*wag31*$_{Mtb}$) by PCR using primers 5'-CTGGTTGCGTTCATCGGTAT-3' and 5'-GAAAACTGGCGCGTGTCC-3'. The concentration of the resulting PCR products was

compared by gel electrophoresis. The 16sRNA gene was used as a control through the experiment.

Thin-layer chromatography (TLC) analysis

Cultures of all the *M. smegmatis* strains were grown to stationary phase (OD_{600} = 2.6), before the cells were pelleted by centrifugation at 3,500 x *g* for 10 min. Supernatants were discarded and the pellets were stored at -20°C overnight. Lipids were extracted by dissolving the pellets in 15 ml of $CHCl_3$:CH_3OH (2:1) per gram of dry material. The mixture was incubated in a water bath at 56°C for 18 hours. After incubation, the samples were centrifuged and the supernatants, containing lipids, were removed and saved. The extraction procedure was repeated one more time. The supernatants of both extractions were combined and left in a fume hood until all the liquid evaporated. The dried lipids were re-dissolved in 1 ml of $CHCl_3$:CH_3OH (2:1) and stored at room temperature until further use. Twenty µl of each sample of lipids were spotted on TLC silica gel plates (Merck). The lipids were developed in a solvent system containing $CHCl_3$:CH_3OH:H_2O (65:25:4). The lipids were derivatized in 5% phosphomolybdic acid in ethanol or in 10% H_2SO_4 in ethanol and baked at 110°C for 20 min.

Antibiotic susceptibility

The antibiotic susceptibility of all *M. smegmatis* strains was determined against kanamycin (0 – 20 µg/ml), isoniazid (0 – 30 µg/ml), and streptomycin (0 – 500 µg/ml)

by the microdilution minimal inhibitory concentration (MIC) method (35). Cells grown to early logarithmic phase (OD_{600} 0.2 – 0.3) were diluted a thousandth fold and 50-µl aliquots were mixed with the different concentrations of the antibiotics on a 96-well round-bottom plate. The plates were placed in plastic bags and incubated at 37°C for 2-3 days. Results were read either as growth or no growth in each well. The lowest concentration of each antibiotic that showed no growth was designated the MIC for that *M. smegmatis* strain.

Infection of macrophages

THP-1 macrophages (ATCC No.TIB-202) were grown on 24–well cell culture plates with RPMI-1640 medium (ATCC Cat. No. 30-2001) supplemented with 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. Cells were incubated in Bio-Bags Type C (Becton, Dickinson and Company, NJ, USA) at 37°C. The Bio-Bags provided an atmosphere of 95% air and 5% CO_2 which is recommended to culture these cells. Prior to infection, macrophages were activated with 16 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (33) for 24 hrs. Following TPA activation, macrophages were infected with the different strains *M. smegmatis* at a multiplicity of infection (MOI) of 10:1. Three hours later, gentamicin (200 µg/ml) was added to each well to eliminate any mycobacterial cell that was not ingested by the macrophages. At 5 hours post infection, the culture medium was removed from each well and stored at -80°C for further cytokine analysis. Fresh medium was added to the cells and incubation continued for 4 more days. During this period, every 24 h the culture media was removed from designated wells and stored at -80°C for further use. In addition, the macrophage

monolayers from these wells were harvested and used for intracellular survival assays (26).

Cytokine analysis and intracellular survival

Cytokine production by infected macrophages was assessed by colorimetric enzyme-linked immunosorbent assays (Endogen, Thermo). The cytokines evaluated were: IL-6, IL-10, IFN-γ and TNF-α. The assays were carried out following the manufacturer's protocol. Taking in account that the culture medium was replaced at 5 h after infection, results obtained for the 5-hour time point were added to the results of the rest of the time points (24 – 96 h), in order to include in the results any cytokine production that occurred during phagocytosis.

Macrophage monolayers were harvested at each time point by adding 500 ml of phosphate-buffered saline (PBS) + 0.25% Tween 80. The cells were scrapped off the walls and transferred to a 1.5 ml plastic microfuge tube. These preparations were probe sonicated for 20 seconds to release any ingested mycobacteria. After sonication, serial dilutions were prepared and plated on Middlebrook 7H11 plates with hygromycin B (50 mg/ml) (26).

III. RESULTS

a. Identification of Wag31$_{Mtb}$

Previous studies have shown that Rel$_{Mtb}$ is involved in the regulation of more than 150 genes, including virulence factor and antigens (9). In order to identify some of these antigens potentially regulated by Rel$_{Mtb}$, protein lysates of H37Rv and H37Rv*Δrel* were compared by Western blot analysis, using a polyclonal antibody produced against *M. tuberculosis* H37Rv whole cell lysate. This polyclonal antibody was first pre-absorbed with a H37Rv*Δrel* whole cell lysate in order to reduce the levels of antigens that were present in both H37Rv and H37Rv*Δrel* cells. By reducing levels of common antigens in this way, only antigens differentially expressed in either strain would be more prominent. The result of this Western blot comparison was a 32-kDa protein band shown to be expressed at higher levels in H37Rv, compared to the H37Rv*Δrel* strain (Figure 3.1). The corresponding protein band was excised from a 10% protein gel and analyzed by matrix assisted laser desorption/ionization. This protein was identified as Wag31$_{Mtb}$, the product of the *M. tuberculosis* gene *Rv2145*.

b. *M. tuberculosis* Wag31 could be expressed in *M. smegmatis*

To confirm the identity of the protein band as Wag31, *Rv2145* was amplified from *M. tuberculosis* genomic DNA and cloned into the shuttle vector pOLYG (14). mc^{2}155 and mc^{2}155*Δrel* strains of *M. smegmatis* were transformed with this plasmid construct and then analyzed by Western blot, using the pre-absorbed polyclonal antibody

described above. The Western blot revealed a protein band of the same molecular weight as Wag31$_{Mtb}$ (Figure 3.2), present in the *M. smegmatis* strains transformed with *wag31$_{Mtb}$* but not in the strains carrying only pOLYG. These results demonstrated that the polyclonal antibody reacted with Wag31$_{Mtb}$ without showing any cross-reactivity with the *M. smegmatis* Wag31 homologue.

c. The presence of Rel enhances the expression of *wag31$_{Mtb}$* in *M. smegmatis*

To evaluate the effect of Rel on the expression of *wag31$_{Mtb}$*, RT-PCR was performed on mRNA extracted from *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$ and *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$. Our results indicate that Rel has a positive effect on the expression of *wag31$_{Mtb}$* (Figure 3.3). Two different concentrations of the cDNA of each strain were used in RT-PCR reactions and both showed that in the absence of Rel, the expression of *wag31$_{Mtb}$* by *M. smegmatis* mc^{2}155Δ*rel*/p*wag31*$_{Mtb}$ was lower than the levels expressed by *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb.}$

d. The presence of *wag31$_{Mtb}$* does not have any effect on the growth *M. smegmatis*

Based on previous reports that Wag31$_{Mtb}$ plays an important role in cell division (20, 21), we decided to evaluate if the *M. smegmatis* strains carrying *wag31$_{Mtb}$* (mc^{2}155/p*wag31*$_{Mtb}$ and mc^{2}155*Δrel*/p*wag31*$_{Mtb}$) showed growth differences when compared with the strains transformed with the vector alone (mc^{2}155/pOLYG and mc^{2}155*Δrel*/pOLYG). For this experiment, the four strains were grown in Middlebrook

7H9 medium + 0.2% glycerol + 0.05% Tween 80 to stationary phase. All strains showed the same growth rate regardless of whether *wag31*$_{Mtb}$ and/or *rel* were present (Figure 3.4). It could be argued that *M. smegmatis* mc^{2}155*wag31*$_{\text{Mtb}}$ did show a higher OD_{600} during most of the stationary phase, but this difference is too subtle to represent a significant finding.

e. The expression of Wag31$_{\text{Mtb}}$ by *M. smegmatis* strains correlates with differences in colony morphology

When grown on Middlebrook 7H11 agar, both *M. smegmatis* strains carrying the *wag31*$_{Mtb}$ gene (mc^{2}155/p*wag31*$_{\text{Mtb}}$ and mc^{2}155*Δrel*/p*wag31*$_{\text{Mtb}}$) show a significantly smoother appearance than the strains not carrying this gene (Figure 3.5). Furthermore, the *M. smegmatis* mc^{2}155/p*wag31*$_{\text{Mtb}}$ colonies appear flatter, particularly around the edges (Figure 3.5 C) compared to the *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{\text{Mtb}}$ colonies (Figure 3.5 D), suggesting that the presence of Rel influences the level of *wag31*$_{Mtb}$ expression in the cell.

f. Wag31$_{\text{Mtb}}$ does not appear to have an effect on the production of cell wall lipids

The differences in colony morphology described above, prompted us to analyze the lipid profiles of the four *M. smegmatis* strains (mc^{2}155/p*wag31*$_{\text{Mtb}}$, mc^{2}155*Δrel*/p*wag31*$_{\text{Mtb}}$, mc^{2}155/pOLYG and mc^{2}155*Δrel*/pOLYG) by TLC. Lipids were extracted from *M. smegmatis* strains grown to stationary phase in Middlebrook 7H9

medium + 0.2% glycerol + 0.05% Tween 80, and spotted on TLC plates. The plates were developed with $CHCl_3$:CH_3OH:H_2O (65:25:4) and the lipids derivatized with 5% phosphomolibdic acid in ethanol (Figure 3.6 A) or 10% H_2SO_4 in ethanol (Figure 3.6 B). The results do not indicate any detectable differences in the lipid profiles of the four strains.

g. Cytokine production by human macrophages infected with *M. smegmatis*

Based on the antigenicity of $Wag31_{Mtb}$, we decided to explore its effect on cytokine production by human macrophages (26). For this experiment, THP-1 macrophages were infected with the four strains of *M. smegmatis* and the cytokine production induced by each strain was examined. The panel of cytokines analyzed included both pro-inflammatory (IL-6 and IFN-γ), as well as anti-inflammatory (TNF-α and IL-10) cytokines (13, 28).

Our results suggest that for IL-6, *M. smegmatis* $mc^2155\Delta rel$/p$wag31_{Mtb}$ induced higher levels of this cytokine than the rest of the strains, with the maximum difference reached at 48 hrs post infection (Figure 3.7). A similar trend was seen for TNF-α production. In this case, *M. smegmatis* mc^2155/p$wag31_{Mtb}$ also induced more TNF-α at 72 and 96 h post-infection; however, the levels of TNF-α induced by *M. smegmatis* mc^2155/p$wag31_{Mtb}$ were approximately 20% lower than those induced by *M. smegmatis* $mc^2155\Delta rel$/p$wag31_{Mtb}$ at the same time points (Figure 3.8).

The production of IFN-γ did not seem to be influenced by any of the *M. smegmatis* strains (Figure 3.9). Only at 96 h post infection did the cells infected with *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$ seem to have produced a slightly higher amount of IFN-γ than the cells infected with the other strains. Similar results were observed for IL-10 production (Figure 3.10), except that the small increase of IL-10 levels by cells infected with *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$ was seen at the 72 hrs time point.

h. Wag31$_{Mtb}$ enhances the intracellular survival of *M. smegmatis* inside THP-1 macrophages

To evaluate the intracellular survival of *M. smegmatis* carrying the *wag31*$_{Mtb}$ gene, THP-1 macrophages were infected with the four *M. smegmatis* strains. At different time points, the macrophages were lysed to release intracellular mycobacteria. These preparations were plated on Middlebrook 7H11 agar + hygromycin B and incubated at 37°C for 4 days. Only the *M. smegmatis* strains carrying *wag31*$_{Mtb}$ appeared to have survived inside the macrophages (Figure 3.11). In addition to intracellular survival, these results could also reveal differences in the uptake of *M. smegmatis* by THP-1 macrophages. In this regard, Wag31$_{Mtb}$ seems to induce or facilitate the ingestion of *M. smegmatis* by THP-1 cells.

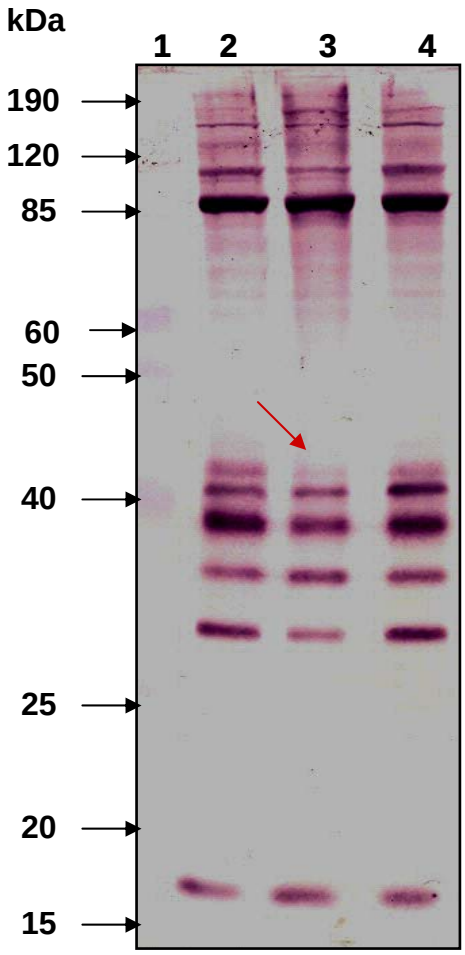

Figure 3.1. Western-blot comparison of protein lysates of H37Rv and H37Rv*Δrel*. Proteins were run on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. A polyclonal antibody produced against *M. tuberculosis* H37Rv whole cell lysate and pre-absorbed with H37Rv*Δrel* was used as the primary antibody. Lane 1. Pre-stained protein ladder. Lane 2. H37Rv protein whole cell lysate. Lane 3. H37RvΔrel_{Mtb} protein whole cell lysate. Lane 4. H37RvΔrel_{Mtb}*attB::*rel_{Mtb}. The red arrow indicates the location of the $_{Wag31Mtb}$ band.

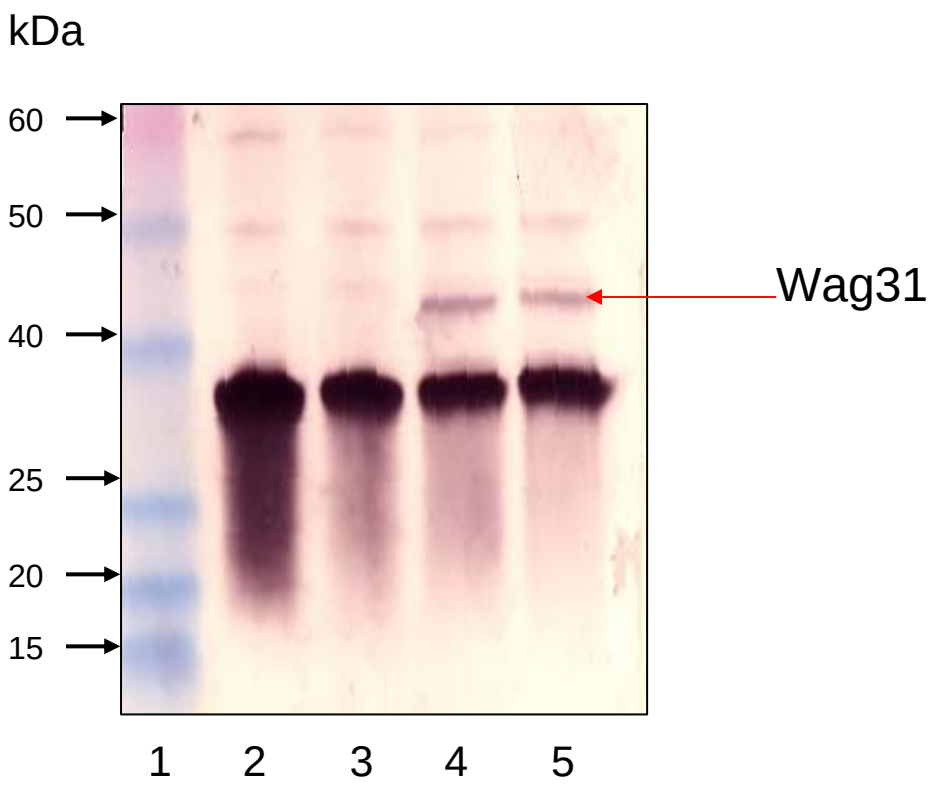

Figure 3.2. Expression of *M. tuberculosis* Wag31$_{Mtb}$ in *M. smegmatis*. The *M. tuberculosis wag31* was cloned into *M. smegmatis* mc^{2}155 and *M. smegmatis* mc^{2}155*Δrel*. Lane 1. Pre-stained protein ladder. Lane 2. *M. smegmatis* mc^{2}155. Lane 3. *M. smegmatis* mc^{2}155*Δrel*. Lane 4. *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$. Lane 5. *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$.

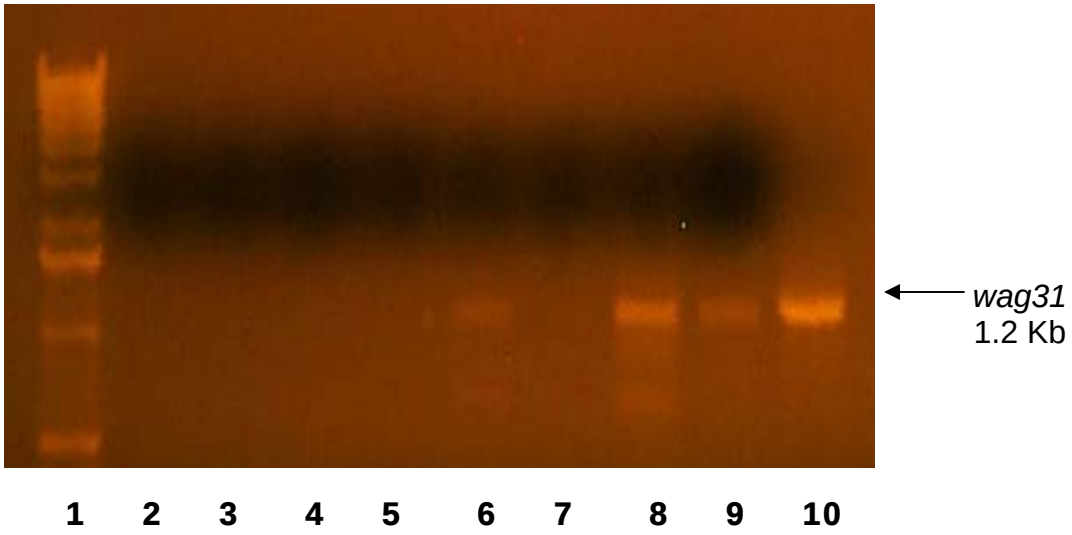

Figure 3.3. Expression levels of *wag31*$_{Mtb}$ mRNA in *M. smegmatis* strains. RNA extracted from *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$ and *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$ was used in RT-PCR reactions to detect RNA expression of *wag31*$_{Mtb}$. Lane 1. 1 Kb DNA ladder. Lan2. 3 – 5, negative controls, no reverse transcriptase. Lane 6. 1:50 dilution *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$. Lane 7. 1:50 dilution *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$. Lane 8. 1:2 dilution *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$. Lane 9. 1:2 dilution *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$. Lane 10. Positive control *M. tuberculosis* genomic DNA. RT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

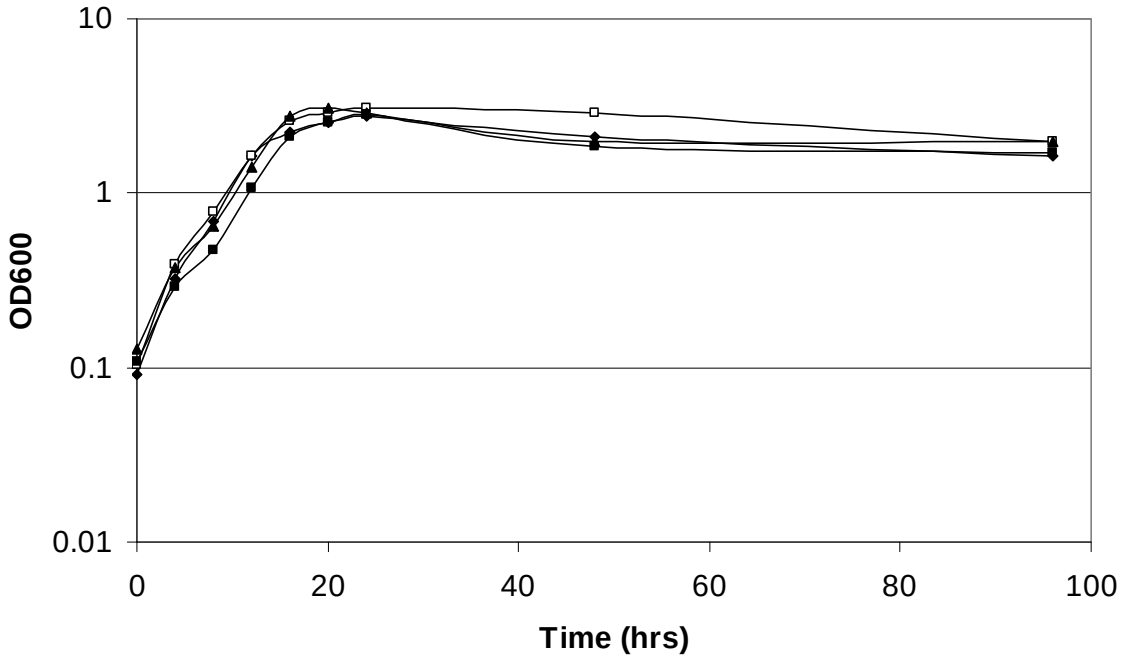

Figure 3.4. Growth of *M. smegmatis* strains. *M. smegmatis strains* were grown in Middlebrook 7H9 medium + 0.2% glycerol + 0.05% Tween 80 for 4 days. OD_{600} readings were taken every four hours during the logarithmic phase and then every 24 hrs during the stationary phase. ♦ *M. smegmatis* mc^2155/ pOLYG. □ *M. smegmatis* mc^2155/p*wag31*$_{Mtb}$. ▲ *M. smegmatis* mc^2155*Δrel:*pOLYG ■ *M. smegmatis* mc^2155*Δrel*/p*wag31*$_{Mtb}$.

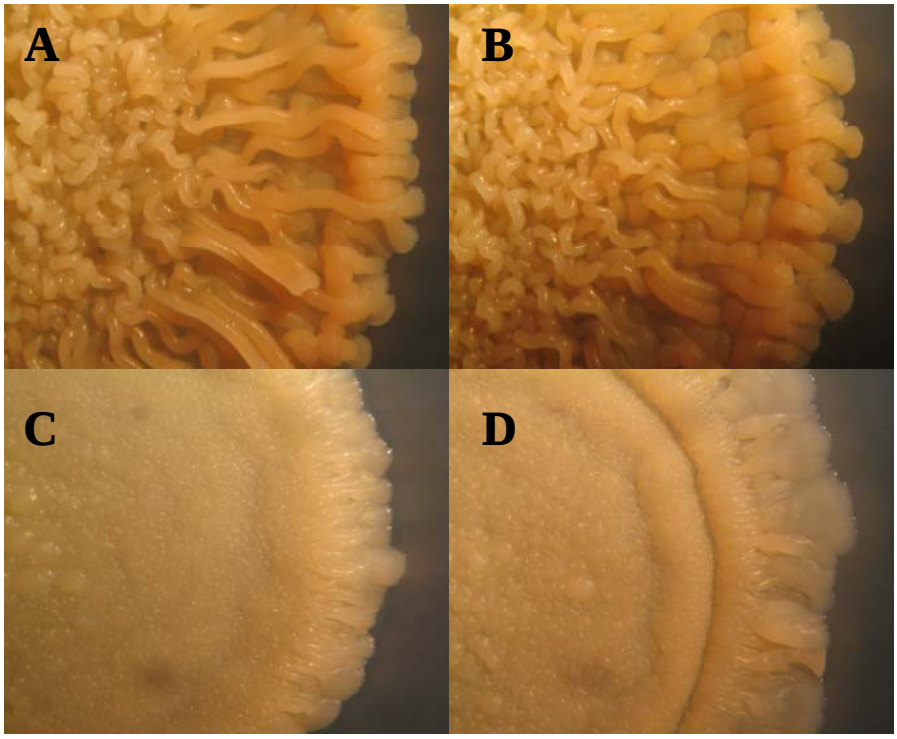

Figure 3.5. Colony morphology of *M. smegmatis* strains. *M. smegmatis strains* were grown in Middlebrook 7H11 medium + 0.2% glycerol for 4 days. **A.** *M. smegmatis* mc²155/ pOLYG **B.** *M. smegmatis* mc²155*Δrel*/pOLYG **C.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **D.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

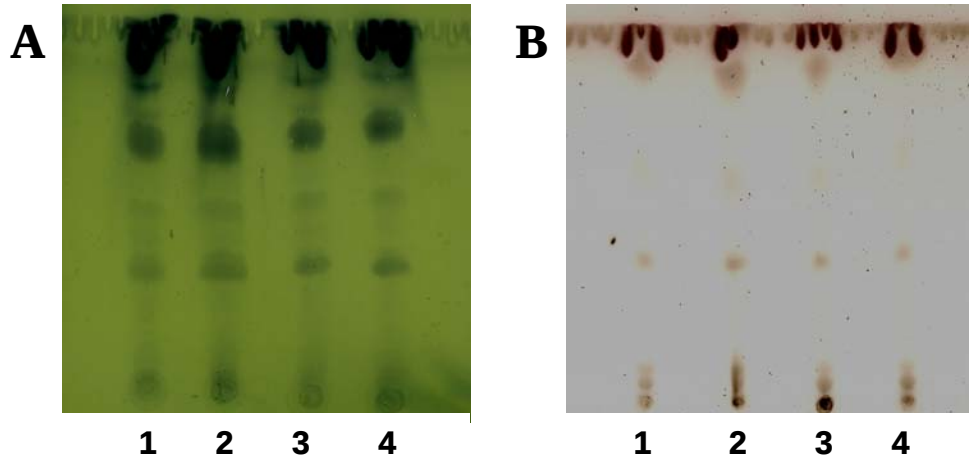

Figure 3.6. Thin-layer chromatography of *M. smegmatis* lipids. Polar lipids were extracted from *M. smegmatis* strains grown to stationary phase in Middlebrook 7H9 medium + 0.2% glycerol + 0.05% Tween 80. The lipids were spotted on TLC plates and developed with $CHCl_3$:CH_3OH:H_2O (65:25:4). Lipids were then derivatized with **A.** 5% phosphomolibdic acid in ethanol or **B.** 10% H_2SO_4 in ethanol. Derivatization was completed by heating the plates at 120°C for 30 min. **1.** *M. smegmatis* mc^{2}155/pOLYG **2.** *M. smegmatis* mc^{2}155/p*wag31*$_{Mtb}$ **3.** *M. smegmatis* mc^{2}155*Δrel*/pOLYG **4.** *M. smegmatis* mc^{2}155*Δrel*/p*wag31*$_{Mtb}$

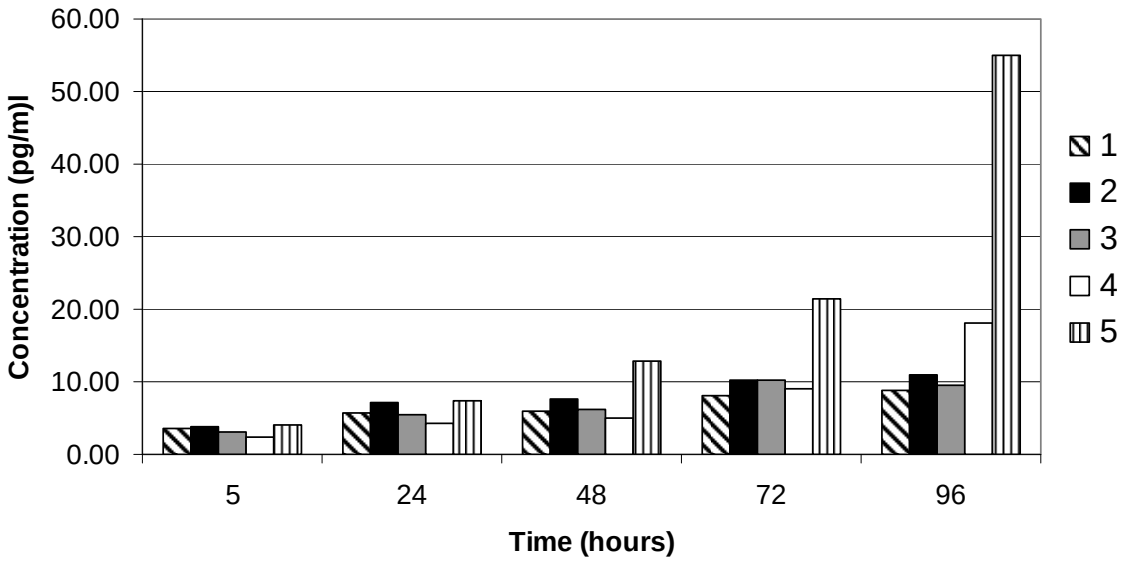

Figure 3.7. Interleukin-6 (IL-6) production by THP-I macrophages infected with *M. smegmatis*. 1. Uninfected macrophages. **2.** *M. smegmatis* mc²155/pOLYG. **3.** *M. smegmatis* mc²155*Δrel*/pOLYG **4.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **5.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

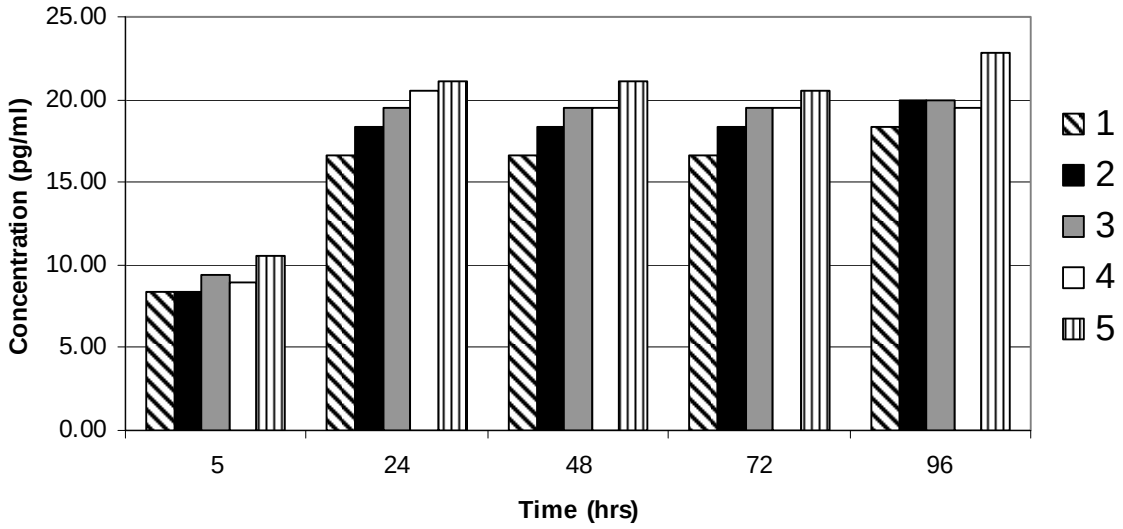

Figure 3.8. **Interferon gamma (IFN-γ) production by THP-I macrophages infected with *M. smegmatis*.** **1.** Uninfected macrophages. **2.** *M. smegmatis* mc²155/ pOLYG. **3.** *M. smegmatis* mc²155*Δrel*/pOLYG **4.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **5.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

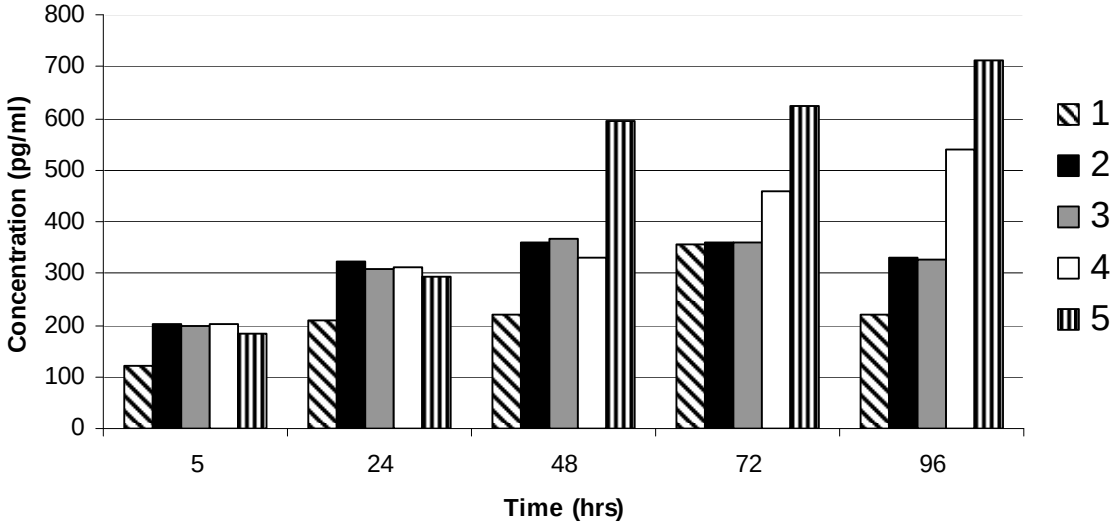

Figure 3.9. **Tumor necrosis factor alpha (TNF-α) production by THP-I macrophages infected with *M. smegmatis*. 1.** Uninfected macrophages. **2.** *M. smegmatis* mc²155/pOLYG. **3.** *M. smegmatis* mc²155*Δrel*/pOLYG **4.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **5.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

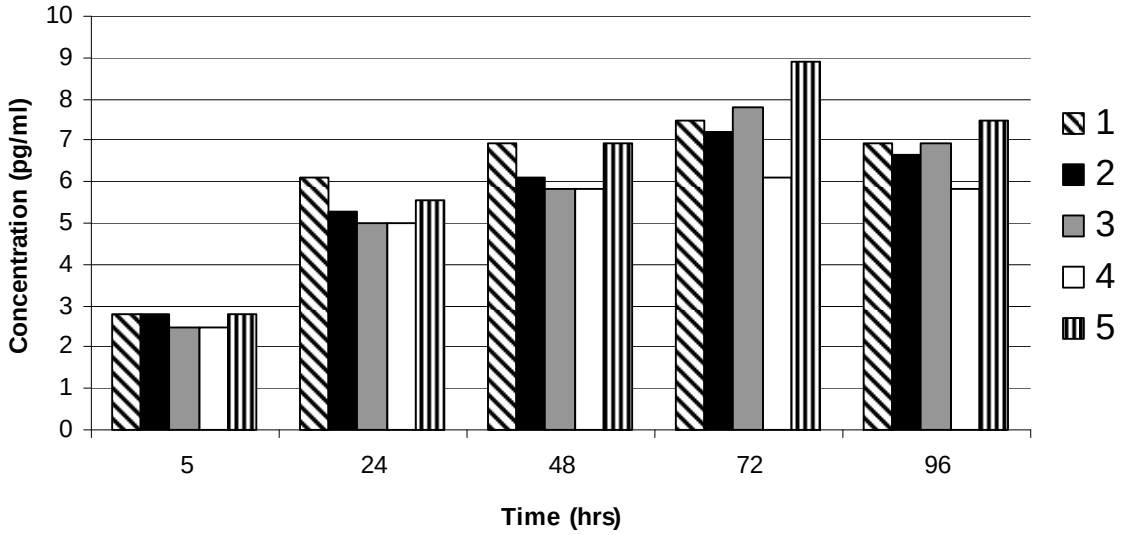

Figure 3.10. Interleukin-10 (IL-10) production by THP-I macrophages infected with *M. smegmatis*. **1.** Uninfected macrophages. **2.** *M. smegmatis* mc²155/pOLYG. **3.** *M. smegmatis* mc²155*Δrel*/pOLYG **4.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **5.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

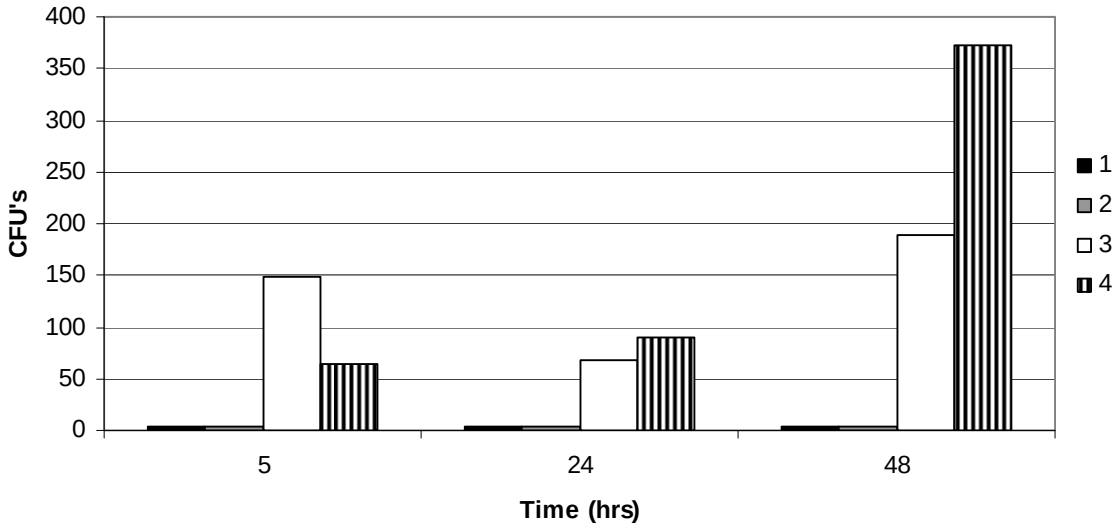

Figure 3.11. Intracellular survival of *M. smegmatis* in THP-1 macrophages. **1.** *M. smegmatis* mc²155/pOLYG. **2.** *M. smegmatis* mc²155*Δrel*/pOLYG **3.** *M. smegmatis* mc²155/p*wag31*$_{Mtb}$ **4.** *M. smegmatis* mc²155*Δrel*/p*wag31*$_{Mtb}$

IV. DISCUSSION

One of the most striking features of *M. tuberculosis* is its ability to remain in a dormant state for long periods of time in infected individuals. This results in a large percentage of infected people that become reservoirs of the organism, with the potential of developing active tuberculosis (7). Furthermore, since all the anti-tuberculosis drugs currently available are only effective against actively replicating cells, dormant bacteria remain unaffected by their action (25). It is not understood how *M. tuberculosis* attains this latent state *in vivo* or how it becomes reactivated after remaining dormant. However, it has been hypothesized that the mechanisms regulating cell division play a crucial role in the process of dormancy and reactivation, likely in response to changes in the host immune system (13, 21, 36). Among these regulatory mechanisms, it has been reported that two serine/threonine kinases are involved in the regulation of cell division in *M. tuberculosis*: PknA and PknB (20, 21). One of the substrates identified for these kinases is $Wag31_{Mtb}$, a homologue of DivIVA of *Bacillus* spp., a protein that is known to play a major role in cell division (21).

By Western blot analysis, we found that wild-type *M. tuberculosis* (H37Rv) produced about four times more $Wag31_{Mtb}$ than a mutant strain lacking Rel_{Mtb} (H37RvΔrel_{Mtb}) (Figure 3.1). Rel_{Mtb} is a global regulator that controls the metabolic changes that *M. tuberculosis* undergoes in response to nutrient starvation and other environmental stresses, a process known as the stringent response (9). The significance of our finding lies in the fact that previous studies showed that H37RvΔrel_{Mtb} is not able to maintain a persistent infection in mice (9). Due to its potential role in cell division,

lower levels of $Wag31_{Mtb}$ in H37RvΔrel_{Mtb} could be, at least in part, responsible for the impairment in long-term viability of this mutant.

To confirm the correlation between lower expression of $Wag31_{Mtb}$ and the absence of Rel_{Mtb}, we cloned the *wag31*$_{Mtb}$ gene into wild-type *M. smegmatis* (mc^2155) and a *rel* mutant strain ($mc^2155\Delta rel$). *M. smegmatis* is routinely used as a surrogate host to express mycobacterial genes of pathogenic species (10), mainly due to its shorter generation time (4 hrs) and its innocuous nature. By Western blot and RT-PCR analysis we found that $mc^2155\Delta rel$/p*wag31*$_{Mtb}$ produced significantly less $Wag31_{Mtb}$ than mc^2155/p*wag31*$_{Mtb}$ (Figures 3.2, 3.3).

Previous microarray analysis showed that Rel_{Mtb} influenced the expression of over 150 genes (9). Although *wag31*$_{Mtb}$ was not among those genes found to be regulated by Rel_{Mtb}, it is important to highlight *ftsZ* and *pknB*, two genes that appeared expressed in a higher concentration in the presence of Rel_{Mtb} (2-fold and 5-fold difference, respectively) than in the absence of it (9). FtsZ is a tubulin-like protein that localizes in the middle of the cell, where it initiates the formation of the Z-ring, a structure that eventually leads to the formation of the septum between daughter cells and later to their separation into individual cells (17).

In the chromosome of *M. tuberculosis*, *wag31*$_{Mtb}$ is located ~ 400 bp downstream *ftsZ* (Figure 3.12). However, *wag31*$_{Mtb}$ appears to have its own promoter and therefore it is unlikely that the lower expression of *wag31*$_{Mtb}$ in H37RvΔrel is due to a polar effect of the reduction of *ftsZ* in the absence of Rel_{Mtb}. The role of $Wag31_{Mtb}$ in cell division is to

direct the assembly of FtsZ and the Z-ring to the middle of the cell (17, 21). Therefore it is possible that the production of $Wag31_{Mtb}$ depends on the levels of FtsZ produced. Thus the absence of Rel_{Mtb} in H37Rv*Δrel* which has been demonstrated to decrease the expression of *ftsZ*, might also be indirectly responsible for the lower production of Wag31 in H37Rv*Δrel*. To test this hypothesis it would be necessary to increase the levels of FtsZ independently of Rel_{Mtb} in H37Rv*Δrel* and measure the levels of $Wag31_{Mtb}$. If the levels of Wag31 increase as more FtsZ is produced, it would indicate that in H37RvΔ*rel*, the absence of Rel has an indirect effect in the production of $Wag31_{Mtb}$. Conversely, if the levels of $Wag31_{Mtb}$ remain low even as FtsZ levels are increased, it could indicate that Rel has a direct effect in the expression of $Wag31_{Mtb}$ or that $Wag31_{Mtb}$ is regulated by another protein that might be in turn regulated by Rel_{Mtb}.

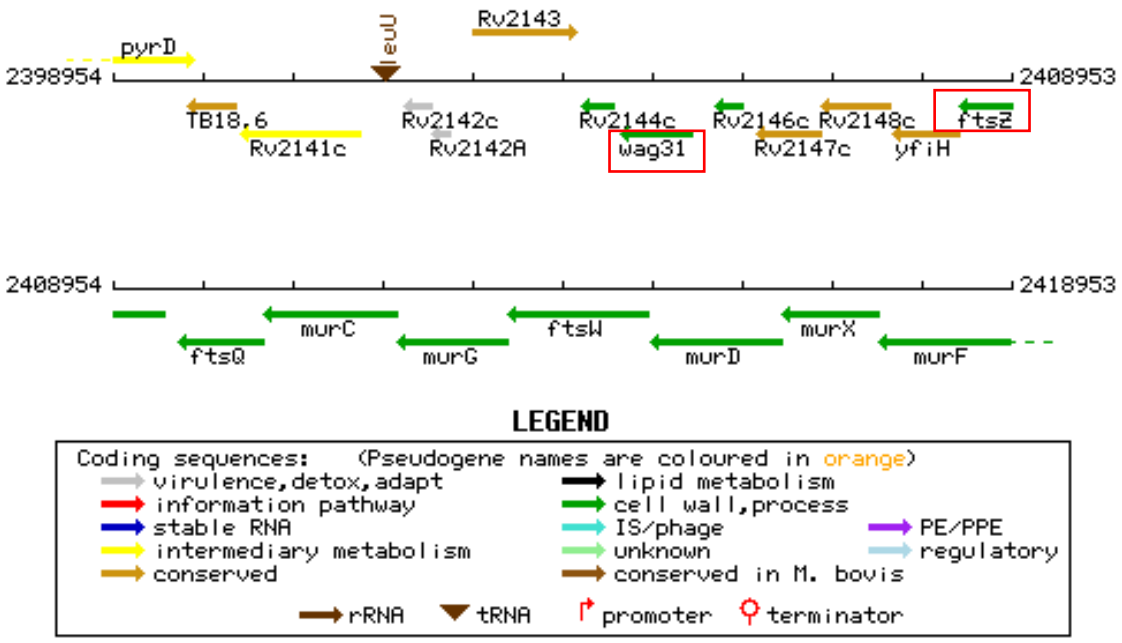

Figure 3.12.Region of *M. tuberculosis* chromosome where *ftsZ* and *wag31* (red boxes) are located (34).

As previously described, $Wag31_{Mtb}$ is the substrate of the serine/threonine kinase PknB (20, 21). *pknB* expression appeared significantly higher (5-fold) in H37Rv than in H37Rv*Δrel* (9). It has been reported that PknB is essential for sustaining growth in several species of mycobacteria (12). PknB is expressed mainly during exponential growth and inside human macrophages infected with *M. tuberculosis* (20, 32). This suggests that PknB could be involved in survival of *M. tuberculosis* inside the host. Based on these data, it is possible that the inability of H37Rv*Δrel* to survive for long periods in infected mice is due to the lower expression of PknB and its substrate, $Wag31_{Mtb}$.

Based on previous studies, in which H37Rv*Δrel* had an impaired growth in comparison with H37Rv when grown in liquid cultures (27), we were expecting to see similar results with the $mc^{2}155\Delta rel$ strain. However, we did not see any difference in the growth of any of the four *M. smegmatis* strains employed regardless of the introduction of $wag31_{Mtb}$ in a background containing or lacking Rel (Figure 3.4). This discrepancy could indicate that despite using *M. smegmatis* routinely as a model organism for the expression of *M. tuberculosis* genes, they are still two distinct species and therefore, there are differences in the physiology of both organisms. The fact that *M. smegmatis* has a larger genome that *M. tuberculosis* indicates that defects that cause growth impairment in the latter could be compensated by other mechanisms in the former.

It has been reported that upon entering stationary phase, a *M. smegmatis Δrel* mutant experiences sharper decreases in cell density compared to the wild-type strain (10, 24). We did not see this difference either, although the length of our growth experiments was considerably shorter (4 days) than those used in their study (> 14 days).

By extending our observations, particularly to the mid-stationary phase, it is possible that our results would be more in accord to previous reports.

An interesting feature of the two strains expressing the *wag31*$_{Mtb}$ gene is the smoothness of their colonies (Figure 3.5). *M. smegmatis* colonies, as well as most mycobacterial species, are usually rough. Smooth variants have been associated with sliding motility on agar, ability to form biofilms, and differences in virulence (1, 31). In other species of mycobacteria, it has been reported that smooth colonies contain lipooligosacharides and glycopeptidolipids that are absent from rough colonies (2, 18). More recently, it was reported that colonies of several species of rapid-growing mycobacteria did not produce glycopeptidolipids but were still smooth and showed sliding motility (1). The authors found that although glycopeptidolipids were absent, all smooth colonies produced a long-chain saturated fatty acid polyester, that was absent from the rough variants of the same species. By TLC analysis we were not able to detect any difference in the total lipid profile of our four *M. smegmatis* strains (Figure 3.6). However, it is possible that more specific extraction techniques that aim at particular components, like fatty acid polyesters, would demonstrate differences in cell wall composition that would explain the variation observed in the texture of the colonies.

Wag31 was originally identified as the mycobacterial Antigen 84 in *M. bovis* and later in *M. tuberculosis* (6). It has been proposed that *M. tuberculosis* regulates the expression of its antigens to modulate the host immune response (15, 22, 29). We analyzed the effect of Wag31$_{Mtb}$ in the production of cytokines (IL-6, IFN-γ, TNF-α, IL-10) by THP-1 macrophages. We decided to perform these experiments using *M.*

smegmatis because this is a non-pathogenic mycobacterial species and it grows significantly faster than *M. tuberculosis*. Our results indicate that the production of IL-6 and TNF-α is induced by Wag31 (Figures 3.7, 3.8). IL-6 is a cytokine involved in inflammation, hematopoiesis and T-cell differentiation (28). As a pro-inflammatory cytokine, it is probably induced by *M. tuberculosis* to attract more cells to the infection site (13). It is believed that TNF-α has various roles in the immune and pathologic responses to tuberculosis (13). TNF-α is important in granuloma formation (28) and its production by macrophages, dendritic cells, and T-cells is induced by *M. tuberculosis* (13). Our findings present Wag31 as one tuberculosis antigen responsible for the induction of IL-6 and TNF-α, two cytokines involved in the process of inflammation and granuloma formation, which are key features of tuberculosis pathogenesis.

Conversely, IL-10 and IFN-γ levels did not seem affected by the presence of Wag31 (Figures 3.9, 3.10). IL-10 has an anti-inflammatory effect and therefore is not induced by *M. tuberculosis* in the initial stages of tuberculosis (13). IFN-γ is a macrophage activator with a key role in the control of tuberculosis infection (13, 28). Although the production of IFN-γ varies considerably between patients, it has been reported that patients with active tuberculosis have lower levels of this cytokine (23, 27). Our results however, indicate that Wag31 does not have an effect in inhibiting the production of IFN-γ.

An interesting finding in our experiments was that both wild-type and *Δrel M. smegmatis* strains carrying $wag31_{Mtb}$ appeared to be able to survive and replicate inside human macrophages, while the two strains containing the vector alone were not able to

survive at all (Figure 3.11). We believed that besides the ability to survive inside macrophages, the expression of $Wag31_{Mtb}$ by *M. smegmatis* also causes the mycobacterial cells to be more readily phagocytized by macrophages. As an antigen, it is possible that one of Wag31 functions is to help in the opsonization of mycobacterial cells. This would facilitate their uptake by macrophages, where they can replicate while escaping the immune system surveillance. To evaluate this hypothesis, it would be necessary to perform macrophage uptake experiments including opsonizing agents (i.e. human serum) as controls.

Overall, this study has contributed to laying the groundwork for further characterization of the role of $Wag31_{Mtb}$ in virulence and persistence of *M. tuberculosis*. Several features of Wag31 have been discovered that can be explored further in order to clarify its role in *M. tuberculosis* and other mycobacterial species. The most interesting findings are the opsonizing capabilities of Wag31 and its influence in cytokine production, which to our knowledge, have not been studied to date. Based upon its essential role in *M. tuberculosis*, $Wag31_{Mtb}$ could be an attractive target for the development of new antimycobacterial drugs.

V. REFERENCES

1. **Agusti, G., O. Astola, E. Rodriguez-Guell, E. Julian, M. Luquin.** 2008. Surface spreading motility shown by a Group of phylogenetically related, rapidly growing pigmented mycobacteria suggests that motility is a common property of mycobacterial species but is restricted to smooth colonies. *J. Bacteriol.* **190**(2):6894-6902.

2. **Belisle, J. T., P. Brennan.** 1989. Chemical basis of rough and smooth variation in mycobacteria. *J. Bacteriol.* **171**(6):3465-3470.

3. **Chauhan, A., H. Lofton, E. Maolney, J. Moore, M. Fol, M. V. V. S. Madiraju, M. Rajagopalan.** 2006. Interference of *Mycobacterium tuberculosis* cell division by Rv2719c, a cell wall hydrolase. *Mol. Microbiol.* **62**(1):132-147.

4. **Chen, Y., D. E. Anderson, M. Rajagopalan, H. P. Erickson.** 2007. Assembly dynamics of *Mycobacterium tuberculosis* FtsZ. *J. Biol. Chem.* **282**(38):27736-27743.

5. **Mshana, R. N., O. Closs, M. Harboe.** 1979. Antibody response in rabbits to *Mycobacterium bovis* BCG. *Scand. J. Immunol.* **9**:175-182.

6. **Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, M. Magnusson.** 1980. The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunoelectrophoresis: a reference system. *Scand. J. Immunol.* **12**:249-263.

7. **Co, D. O., L. H. Hogan, S. I. Kim, M. Sandor.** 2004. Mycobacterial granulomas: keys to a long-lasting host-pathogen relationship. *Clin. Immunol.* **113**:130-136.

8. **Dahl, J. L., J. Wei, J. W. Moulder, S. Laal, R. L. Friedman.** 2001. Subcellular localization of the intracellular survival-enhancing Eis protein of *Mycobacterium tuberculosis. Infect. Immun.* **69**(7):4295-4302.

9. **Dahl, J. L., C. N. Kraus, H. I. M. Boshoff, B. Doan, K. Foley, D. Avarock, G. Kaplan, V. Mizrahi, H. Rubin, C. E. Barry III.** 2003. The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc. Natl. Acad. Sci. USA*. **100**:10026-10031.

10. **Dahl, J. L., K. Arora, H. I. Boshoff, D. C. Whiteford, S. A. Pacheco, O. J. Walsh, D. Lau-Bonilla, W. B. Davis, A. Garza.** 2005. The *relA* homolog of *Mycobacterium smegmatis* affects cell appearance, viability, and Gene Expression. *J. Bacteriol.* **187**(7):2439-2447.

11. **Dziadek, J., S. A. Rutherford, M. V. Madiraju, M. A. L. Atkinson, M. Rajagopalan.** 2003. Conditional expression of *Mycobacterium smegmatis ftsZ*. An essential cell division gene. *Microbiology*. **149**:1593-1603.

12. **Fernandez, P., B. Saint-Joanis, N. Barilone, M. Jackson, B. Gicquel, S. T. Cole, P. M. Alzari.** 2006. The ser/thr protein kinase PknB is essential fro sustaining mycobacterial growth. *J. Bacteriol.* **188**(22):7778-7784.

13. **Flynn, J. L., J. Chan.** 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* **19**:93-129.

14. **Garbe, T. R., J. Barathi, S. Barnini, Y. Zhang, C. Abou-Zeid, D. Tang, R. Mukherjee, and D. B. Young.** 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. *Microbiology*. **140:**133-138.

15. **Gehring A. J., K. M. Dobos, J. T. Belisle, C. V. Harding, W. H. Boom.** 2004. *Mycobacterium tuberculosis* LprG (*Rv1411c*): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J. Immunol.* **173**:2660-2668.

16. **Hermans, P. W. M., F. Abebe, V. I. O. Kuteyi, A. H. J. Kolk, J. E. R. Thole, M. Harboe.** 1995. Molecular and immunological characterization of the highly conserved antigen 84 from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *Infect. Immun.* **63**(3):954-960.

17. **Hett, E. C., E. J. Rubin.** 2008. Bacterial growth and cell division: a mycobacterial perspective. *Microbiol. Mol. Biol. Rev.* **72**(1):126-156.

18. **Howard, S. T., E. Rhoades, J. Recht, X. Pang, A. Alsup, R. Kolter, C. R. Lyons, T. F. Byrd.** 2006. Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology*. **152**:1581-1590.

19. **Hu, Z., C. Saez, J. Lutkenhaus.** 2003. Recruitment of MinC, an inhibitor of Z-ring formation, to the membrane in *Escherichia coli*: Role of MinD and MinE. *J. Bacteriol.* **185**(1):196-203.

20. **Kang, C. M., D. W. Abbott, S. T. Park,, C. C. Dascher, L. C. Cantley, R. N. Husson.** 2005. The *Mycobacterium tuberculosis* serine/threonine kinases PknA ans PknB: substrate identification and regulation of cell shape. *Genes and Dev*. **19**:1692-1704.

21. **Kang, C. M., S. nyayapathy, J. Y. Lee, J. W. Suh, R. Husson.** 2008. Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. **154**:725-735.

22. **Korf, J., A. Stoltz, J. Verschoor, P. De Baetselier, J. Grooten.**2005. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *Eur. J. Immunol.* **35**:890-900.

23. **Lin, Y., M. Zhang, F. M. Hofman, J. Gong, P. F. Barnes.** 1996. Absence of a prominent Th2 cytokine response in Human tuberculosis. *Infect. Immun.* **64**(4):1351-1356.

24. **Mathew, R., A. K. Ojha, A. A. Karande, D. Chatterji.** 2004. Deletion of the *rel* gene in *Mycobacterium smegmatis* reduces its stationary phase survival without altering the cell-surface associated proteins. *Curr. Sci.* **86**(10):149-153.

25. **Palomino, J. C., S. C. Leao, V.. Ritacco.** 2007. Tuberculosis: from basic science to patient care. Available from: http://www.tuberculosistextbook.com/

26. **Post, F. A., C. Manca, O. Neyrolles, B. Ryffel, d. B. Young, G. Kaplan.** 2001. *Mycobacterium tuberculosis* 19-kilodalton lipoprotein inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages *in vitro.* Infect. Immun. **69**(3):1433-1439.

27. **Primm, T. P., S. J. Andersen, V. Mizrahi, D. Avarock, H. Rubin, C. E. Barry III.** 2000. The stringent response of *Mycobacterium tuberculosis* required for long-term survival. *J. Bacteriol.* **182**(17):4889-4898.

28. **Raja, A.** 2004. Immunology of tuberculosis. *Indian J. Med, Res.* **19**:213-232.

29. **Rao, V., N. Dhar, A. K. Tyagi.** 2003. Modulation of host immune responses by overexpression of immunodominant antigens of *Mycobacterium tuberculosis* in Bacille Calmette-Guerin. *Scand. J. Immunol.* **58**:449-461.

30. **Sassettti, C. M., D. Boyd. E. Rubin.** 2003. Genes induced for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **48**(1):77-84.

31. **Schorey, J. S., L. Sweet.** 2008. The mycobacterial glycopeptidolipids: structure, function, and their role in pathogenesis. *Glycobiol.* **18**(11):832-841.

32. **Singh, A., Y. Singh, R. Pine, L. Shi, R. Chandra, K. Drlica.** 2006. Protein kinase I of *Mycobacterium tuberculosis*: cellular localization and expression during infection of macrophage-like cells. *Tuberculosis*. **86**:28-33.

33. **Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, K. Tada.** 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* **42**:1530-1536.

34. **Tuberculist** [Internet]. France: Institut Pasteur; c1999-2008. Available from: http://genolist.pasteur.fr/TubercuList/

35. **Wallace Jr., R. J., D. R. Nash, L.C. Steele, V. Steingrube.** 1986. Susceptibility testing for slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. *J. Clin. Microbiol.* **24**(6):976-981.

36. **Wayne, L. G., L. G. Hayes.** 1996. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of non-replicating persistence. *Infect. Immun.* **64**(6):2062-2069.

37. **Zhang, M., Y. Lin, D. V. Iyer, J. Gong, J. S. Abrams, P. F. Barnes.** 1995. T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. **63**(8):3231-3234.

CHAPTER FOUR

DISCUSSION

I. FOREWORD

The core hypothesis of this work is that Rel_{Mtb}, the coordinator of the stringent response in *M. tuberculosis*, influences the expression of mycobacterial antigens that are involved in the pathogenesis of tuberculosis.

Our goal was to find a correlation between Rel_{Mtb} and the modulation of the host immune response exerted by *M. tuberculosis* through the expression of several antigens. Establishing that link would contribute to the understanding of the mechanisms that *M. tuberculosis* utilizes to subvert the immune system and how those mechanisms are interconnected with the ability of this pathogen to survive for extended periods inside the host (14).

Our research was focused on two antigens: Glutamine synthetase A1 (GlnA1) and Antigen 84 (Wag31). While our results do not comprehensively explain why these two antigens would be modulated by Rel_{Mtb}, or how that modulation will benefit *M. tuberculosis* and its interaction with the host, our findings do provide preliminary data that could be the foundation for further research. In the following pages, we will describe a series of experiments designed to complement and to develop the work presented in this dissertation.

II. GLUTAMINE SYNTHETASE (GlnA1)

In most bacteria, glutamine synthetase (GlnA1) has a central role in nitrogen metabolism, especially in ammonia assimilation and synthesis of glutamine (2). In *M. tuberculosis,* it has been shown that GlnA1 has an essential role for growth *in vitro* and *in vivo* and has a potential involvement in the pathogenesis of tuberculosis (6, 7, 27, 28). Based on this knowledge, the finding that H37RvΔ*rel* produces considerably less GlnA1 is very significant, because it demonstrates that Rel is involved in the regulation of pathogenic determinants.

To further evaluate the impact of the lower expression of GlnA1 in H37Rv*Δrel* and consequentially test the hypothesis that Rel is involved in the pathogenesis of tuberculosis, we propose to address the following issues:

a. Does the low production of GlnA1 prevent H37Rv*Δrel* from arresting phagosome acidification?
b. Does less GlnA1 cause H37Rv*Δrel* to produce less poly-L-glutamic acid/glutamine, (P-L-glx)?

A key element for dealing with these questions is the development of a strain with a decreased expression of *glnA1* that is able to produce similar levels of GlnA1 as H37Rv*Δrel*. To generate such strain, we will take advantage of the inducible acetamidase promoter and an antisense RNA approach (19). This method has been successfully applied in other studies of mycobacterial genes (18, 25). Briefly, in this technique the

glnA1 gene from *M. tuberculosis* wild-type (H37Rv) genomic DNA is PCR amplified in an antisense orientation. The product is then cloned behind the inducible acetamidase promoter. In this way, when the promoter is induced with acetamide, antisense copies of *glnA1* mRNA should be produced, resulting in a decreased level of GlnA1 (19). We will refer to this knockdown strain as H37Rv:p_{anti}*glnA1*.

a. Does the low production of GlnA1 prevent H37Rv*Δrel* from arresting phagosome acidification?

It has been reported that in *M. tuberculosis*, GlnA1 could exert a glutamate dehydrogenase activity, releasing ammonia from glutamate (27). It is also known that GlnA1 is exported in relatively high quantities to the extracellular environment (one third of the total production) (6). These results have led to the hypothesis that this enzyme is involved in preventing the acidification of phagosomes by increasing the levels of ammonia (6). This idea was further supported by the finding that non-pathogenic species of mycobacteria did not export GlnA1 extracellularly (22). Based on these reports, we hypothesized that due to its reduced production of GlnA1, H37Rv*Δrel* is unable to generate enough ammonia to prevent the acidification of the phagosomes. To address this hypothesis we propose the infection of THP-1 macrophages with H37Rv, H37Rv*Δrel* and H37Rv:p_{anti}*glnA1*. This would allow a comparison of the levels of GlnA1 produced *in vitro*, the amount of the enzyme secreted into the phagosomal space by the three strains, and the pH of the phagosomes containing mycobacteria.

Levels of GlnA1 produced *in vitro* will be estimated by Western-blot analysis of whole cell lysates, as described before (Chapter 2). The GlnA1 secreted into the culture media will be concentrated by ultrafiltration (Millipore) and analyzed by Western-blot utilizing an anti-GlnA1 antibody (6).

To measure the levels of GlnA1 released into the phagosomes, it would be necessary to isolate mycobacteria-containing phagosomes from infected macrophages (13). These phagosomes would be probe sonicated to release the ingested bacteria. These preparations would be filtered through a 0.22 μm filter to separate bacteria from the phagosomal material. The contents of the phagosomes would be used to compare levels of GlnA1 secreted by the three mycobacterial strains by Western blot analysis using the anti-GlnA1 antibody.

To measure the intraphagosomal pH, we would use a fluorescent technique that comprises two fluorescent probes: fluorescein (pH sensitive) and rhodamine (pH insensitive). The pH is then estimated by the ratio of fluorescein/rhodamine fluorescence measured by flow cytometry (4, 15).

We would expect to find lower levels of GlnA1 produced and secreted by H37Rv*Δrel* and H37Rv:p_{anti}*glnA1* in comparison with H37Rv, as well as an acidic pH in the phagosomes containing the first two strains, as opposed to a neutral pH in the phagosomes containing H37Rv. Such findings would suggest that GlnA1 plays a role in the modulation of the intraphagosomal pH, and its lower production in H37Rv*Δrel* could affect the intracellular survival of this strain. Conversely, if the levels of secreted GlnA1

are different between the strains but the intraphagosomal pH is similar for the three strains, it would indicate that the concentration of GlnA1 in the phagosome does not affect the pH inside these compartments; consequently the inability of H37Rv*Δrel* to survive inside macrophages must be associated to other factors.

b. Does less GlnA1 cause H37RvΔ*rel* to produce less poly -L-glutamic acid/glutamine, (P-L-glx)?

It has been shown that the production of GlnA1 by pathogenic species of mycobacteria correlates with the presence of a unique polypeptide in their cell wall. This compound has been identified as poly-L-glutamic acid/glutamine (P-L-glx) and it has been reported to be absent in non-pathogenic mycobacteria (10, 28). This has led to the hypothesis that the GlnA1 secreted to the extracellular environment is involved in the synthesis of P-L-glx and that as a result, GlnA1 is linked to mycobacterial virulence (8).

Based on these reports, we propose that the impaired production of GlnA1 in H37RvΔ*rel* would cause a lower production of P-L-glx in the cell walls of this strain. This could help explain the reduced virulence of H37Rv*Δrel* in mice.

In order to test this hypothesis, it is necessary to determine and compare the P-L-glx content in the cell walls of H37Rv, H37Rv*Δrel*, and H37Rv:p_{anti}*glnA1*. It has been established that P-L-glx is associated with the peptidoglycan layer of the cell wall, but it can be extracted by a combination of differential centrifugation, sodium dodecyl sulfate (SDS) extraction, sucrose and Percoll-Tween 80 gradient centrifugation. This technique

renders a band of insoluble, pure P-L-glx (10). The concentration of P-L-glx could then be determined by Western blot analysis using a rabbit polyclonal antibody raised against *M. tuberculosis* whole cell lysate, such as the one described in Chapter 3.

We would expect to find considerably more P-L-glx in H37Rv than in H37Rv*Δrel* and H37Rv:p_{anti}*glnA1*. These results, in combination with the levels of GlnA1 produced and secreted by each of the three strains, would support our hypothesis that the decrease in GlnA1 production, resulting from the absence of Rel in H37Rv*Δrel*, has an impact on the production of virulence determinants such as P-L-glx. The next step would be to perform macrophage infections with the three strains to evaluate if there is a correlation between levels of P-L-glx and intracellular survival.

III. Wag31

wag31 (*Rv2145c*) is an essential gene in *M. tuberculosis* (23), and its product, $Wag31_{Mtb}$ (also known as Antigen 84), is a mycobacterial protein with a crucial role in cell division (9, 12, 17). In our lab, we found that the wild-type strain H37Rv produced significantly more $Wag31_{Mtb}$ than the *rel* mutant, H37Rv*Δrel* (Chapter 3). Because of the antigenic nature of $Wag31_{Mtb}$ and its potential connection with Rel, we decided to evaluate its immunomodulatory potential on the expression of cytokines by human macrophages.

We found that when $wag31_{Mtb}$ was expressed in *M. smegmatis* it induced the expression of IL-6 and TNF-α, two cytokines with significant roles in the pathogenesis of

tuberculosis (5, 21). *M. smegmatis* normally does not have an effect on cytokine induction, indicating that our results were caused by $Wag31_{Mtb}$ (20).

In our experiments, the use of *M. smegmatis* as a surrogate host for the expression of a *M. tuberculosis* gene was very convenient for three main reasons: first, since *M. smegmatis* is arapid growing mycobacteria, it allows to complete experiments in a fraction of the time that it will take by using *M. tuberculosis.* Second, the non-pathogenic nature of *M. smegmatis* facilitates its manipulation. Lastly, it provided us with a system that illustrates how a mycobacterial protein that is conserved across the genus, may have different roles in a pathogenic species than in a non-pathogenic one. Related to this last point, it is important to highlight that the polyclonal antibody that was used to detect $Wag31_{Mtb}$, did not react with the *M. smegmatis* Wag31 homologue (Figure 3. 2, p. 80). This is an interesting finding because although both proteins share an 87% similarity (Figure 4.1), only Wag31 from *M. tuberculosis* reacts with our polyclonal antibody, suggesting that the immunogenic epitope resides in a very small region of the protein. This is supported by the fact that the *M. bovis* Wag31 homologue shares a 100% similarity with $Wag31_{Mtb}$, and it was in *M. bovis* where this antigen was originally identified (3).

Another interesting finding was that both *M. smegmatis* strains carrying the $wag31_{Mtb}$ gene ($mc^{2}155$/p$wag31_{Mtb}$ and $mc^{2}155\Delta rel$/ p$wag31_{Mtb}$), seemed to be able to survive inside infected macrophages, while the strains carrying the vector alone lacked this ability (Figure 3. 11, p. 89). However, it is also possible that these results are a consequence of the ability of THP-1 macrophages to differentially phagocytize each

strain. It could be that the strains carrying the *wag31*$_{Mtb}$ gene were readily phagocytized while the ones carrying the vector alone were not. To test this possibility, first it would be necessary to corroborate our results by performing more macrophage infections using the conditions described in Chapter 3, with the addition of human serum as an opsonizing agent (1, 11).

In previous studies, it has been reported that incubating mycobacterial cells with serum, at least 1 h before infection, increases significantly the uptake of bacteria by macrophages (24). By pre-treating the mycobacterial cells with serum, we would expect to facilitate the phagocytosis of the *M. smegmatis* strains carrying the vector alone (mc^{2}155/pOLYG and mc^{2}155*Δrel*/pOLYG). The objective of this part of the experiment would only be to evaluate the uptake of mycobacteria. After allowing the macrophages to phagocytize bacteria (~ 3 h), the cell monolayers would be washed and treated with gentamycin, to remove any uningested bacteria. Immediately following this step, the macrophages would be lysed to release the phagocytized bacteria. The number of ingested mycobacteria would be calculated either by standard plate count or by directly counting the cells using a hemocytometer.

In the case of mc^{2}155/pOLYG and mc^{2}155*Δrel*/pOLYG, if the macrophages are able to phagocytize serum-treated cells but not untreated ones, it would demonstrate that the uptake of *M. smegmatis* cells requires some type of opsonization. For the *M. smegmatis* strains carrying the Wag31$_{Mtb}$ gene (mc^{2}155/p*wag31*$_{Mtb}$ and mc^{2}155*Δrel*/p*wag31*$_{Mtb}$), we would expect to see the same numbers of ingested cells in both serum-treated and untreated preparations. By combining these results we would be able to

determine if $Wag31_{Mtb}$ facilitates the phagocytosis of *M. smegmatis,* which would be an exciting observation, given that this role has never been reported for Wag31. Once the question of phagocytosis has been addressed, the aspects of intracellular survival and cytokine induction could be further explored.

In addition, it would be interesting to evaluate if other proteins associated with $Wag31_{Mtb}$ and the cell division process (PknA, PknB, FtsZ among others) are also influenced by Rel.

In conclusion, the work presented here provides preliminary data that can be used as the foundation to explore:

a. The role of GlnA1 in the pathogenesis of *M. tuberculosis*, in particular on the production of P-L-glx and as a regulator of the intraphagosomal environment.

b. The potential of $Wag31_{Mtb}$ as an antigen and as inducer of phagocytosis. Both characteristics could be exploited in the development of vaccines and new drugs.

Furthermore, our findings support the hypothesis that Rel_{Mtb} participates in the regulation of a wide variety of genes, including several involved in pathogenesis, antigenicity, and virulence. As such, this work contributes to the field of mycobacteriology by providing more evidence of the significant role of Rel_{Mtb} in the physiology and pathogenesis of *M. tuberculosis*.

```
Score =  417 bits (1071),  Expect = 1e-121, Method: Compositional matrix adjust.
Identities = 217/272 (79%), Positives = 237/272 (87%), Gaps = 12/272 (4%)

  M. smegmatis      1    MPLTPADVHNVAFSKPPIGKRGYNEDEVDAFLDLVENELTRLIEENADLRQRVAELDQEL  60
                         MPLTPADVHNVAFSKPPIGKRGYNEDEVDAFLDLVENELTRLIEEN+DLRQR+ ELDQEL
  M. tuberculosis   1    MPLTPADVHNVAFSKPPIGKRGYNEDEVDAFLDLVENELTRLIEENSDLRQRINELDQEL  60

  M. smegmatis      61   AAARSGAGASSQATSSIPLYEPEPEPAPAPPQPVYEAPAQPAAPQSEDTAVRAARVLSLA  120
                         AA   GAG + QAT +IP YEPEP      P     APA  +A  +E+ A++AARVLSLA
  M. tuberculosis   61   AAG-GGAGVTPQATQAIPAYEPEP----GKP-----APAAVSAGMNEEQALKAARVLSLA  110

  M. smegmatis      121  QDTADRLTSTAKAEADKLLSDARAQAEAMVSDARQTAETTVSEARQRADAMLADAQTRSE  180
                         QDTADRLT+TAKAE+DK+L+DARA AE ++ +AR TA+ TV+EARQRADAMLADAQ+RSE
  M. tuberculosis   111  QDTADRLTNTAKAESDKMLADARANAEQILGEARHTADATVAEARQRADAMLADAQSRSE  170

  M. smegmatis      181  AQLRQAQEKADALQADAERKHSEIMGTINQQRTVLEGRLEQLRTFEREYRTRLKTYLESQ  240
                         AQLRQAQEKADALQADAERKHSEIMGTINQQR VLEGRLEQLRTFEREYRTRLKTYLESQ
  M. tuberculosis   171  AQLRQAQEKADALQADAERKHSEIMGTINQQRAVLEGRLEQLRTFEREYRTRLKTYLESQ  230

  M. smegmatis      241  LEELGQRGSAAPVDSSANSDASGFGQFNRGNN  272
                         LEELGQRGSAAPVDS  N+DA GF QFNRG N
  M. tuberculosis   231  LEELGQRGSAAPVDS--NADAGGFDQFNRGKN  260
```

Figure 4.1. Sequence alignment of Wag31 from *M. smegmatis* and *M. tuberculosis* (16).

IV. REFERENCES

1. **Bjornson, A. B., P. I. Magnafichi, R. D. Schreiber, H. S. Bjornson.** 1987. Opsonization of *Bacteroides* by the alternative complement pathway reconstructed from isolated plasma proteins. *J. Exp. Med.* **164**:777-798.

2. **Brown J.R., Masuchi Y., Robb F.T., Doolittle W.F. 1994.** Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. *J. Mol. Evol.* **38**:566-576.

3. **Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, M. Magnusson.** 1980. The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunoelectrophoresis: a reference system. *Scand. J. Immunol.* **12**:249-263.

4. **Dri, P., G. Presani, S. Perticarari, L. Alberi, M. Prodan, E. Decleva.** 2002. Measurement of phagosomal pH of normal and CGD-like human neutrophils by dual fluorescente flow cytometry. *Cytometry*. **48**:159-166.

5. **Flynn, J. L., J. Chan.** 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* **19**:93-129.

6. **Harth, G., D. L. Clemens, M. A. Horwitz.** 1994. Glutamine synthetase of *Mycobacterium tuberculosis*: Extracellular release and characterization of its enzymatic activity. *Proc. Natl. Acad. Sci. USA.* **91**:9342-9346.

7. **Harth, G., M. A. Horwitz.** 1997. Expression and efficient export of enzymatically active *mycobacterium tuberculosis* glutamine synthetase in *Mycobacterium smegmatis* and evidence that the information for export is contained within the protein. *J. Biol. Chem*. **272**(36):22728-22735.

8. **Harth, G., M. A. Horwitz.** 1999. An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. *J. Exp. Med.* **189**(9):1425-1435.

9. **Hermans, P. W. M., F. Abebe, V. I. O. Kuteyi, A. H. J. Kolk, J. E. R. Thole, M. Harboe.** 1995. Molecular and immunological characterization of the highly conserved antigen 84 from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *Infect. Immun.* **63**(3):954-960.

10. **Hirschfield, G. R., M. McNeil, P. J. Brennan.** 1990. Peptidoglycan-associated polypeptides of *Mycobacterium tuberculosis*. *J. Bacteriol.* **172**(2):1005-1013.

11. **Horwitz, M. A.** 1982. Phagocytosis of microorganisms. *Rev. Infect. Dis.* **4**(1):104-123.

12. **Kang, C. M., S. Nyayapathy, J. Y. Lee, J. W. Suh, R. Husson.** 2008. Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. **154**:725-735.

13. Lonnbro, P., P. Nordenfelt, H. Tapper. 2008. Isolation of bacteria-containing phagosomes by magnetic selection. *BMC Cell Biol.* **9**(35). Available from: http://www.biomedcentral.com/1471-2121/9/35

14. **Manabe, Y. C., W. R. Bishai.** 1999. Latent *Mycobacterium tuberculosis* – persistence, patience, and winning by waiting. *Nat. Med.* **6**(12):1327-1329.

15. **Murphy, R. F., S. Powers, C. R. Cantor.** 1984. Endosome pH measured in single cells by dual fluorescence flow cytometry: Rapid acidification of insulin to pH6. *J. Cell. Biol.* **98**:1757-1762.

16. **National Center for Biotechnology Information. NCBI.** [Internet]. Search: gi:118470637, gi:3161817. Bethesda, MD. U.S. National Library of Medicine; 2008. Available from: http://www.ncbi.nlm.nih.gov/

17. **Nguyen, L., N. Scherr, J. Gatfield, A. Walburger, J. Pieters, C. J. Thompson.** 2007. Antigen 84, an effector of pleiomorphism in *Mycobacterium smegmatis*. *J. Bacteriol.* **189**(21):7896-7910.

18. **Ojha, A. K., T. K. Mukherjee, D. Chatterji.** 2000. High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes the morphology of the bacterium. *Infect. Immun.* **68**(7):4084-4091.

19. **Parish, T., N. G. Stoker.** 1997. Development and use of a conditional antisense mutagenesis system in mycobacteria. *FEMS Microbiol. Lett.* **154**:151-157.

20. **Post, F. A., C. Manca, O. Neyrolles, B. Ryffel, d. B. Young, G. Kaplan.** 2001. *Mycobacterium tuberculosis* 19-kilodalton lipoprotein inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages *in vitro.* Infect. Immun. **69**(3):1433-1439.

21. **Raja, A.** 2004. Immunology of tuberculosis. *Indian J. Med, Res.* **19**:213-232.

22. **Raynaud, C., G. Etienne, P. Peyron, M. A. Laneelle, M. Daffe.** 1998. Extracellular enxymes activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis. Microbiology*. **144**:577-587.

23. **Sassetti, C. M., D. Boyd. E. Rubin.** 2003. Genes induced for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **48**(1):77-84.

24. **Swartz, R. P., D. Naai, C. W. Vogel, H. Yeager Jr.** 1988. Differences in uptake of mycobacteria by human monocytes: a role for complement. *Infect. Immun.* **56**(9):2223-2227.

25. **Triccas, J. A., T. Parish, W. J. Britton, B. Gicquel.** 1998. An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. *FEMS Microbiol. Lett*. **167**:151-156.

26. **Tullius, M. V., G. Harth, M. Horwitz.** 2001. High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than to a protein-specific export mechanism. *Infect. Immun.* **69**(10):6348-6363.

27. **Tullius, M. V., G. Harth, M. Horwitz.** 2003. Glutamine synthetase GlnA1 is essential for growth of *Mycobacterium tuberculosis* in Human THP-1 macrophages and Guinea pigs. *Infect. Immun.* **71**(7):3927-3936.

28. **Wietzerbin, J., F. Lederer, and J.-F. Petit.** 1975. Structural study of the poly-L-glutamic acid of the cell wall of *Mycobacterium tuberculosis* var. hominis, strain brevannes. *Biochem.Biophys. Res. Commun*. **62**:246-252.

APPENDIXES

COLLABORATIVE WORK

APPENDIX A

The *relA* Homolog of *Mycobacterium smegmatis* Affects Cell Appearance, Viability, and Gene Expression

John L. Dahl, Kriti Arora, Helena I. Boshoff, Danelle C. Whiteford, Sophia A. Pacheco, Olaus J. Walsh, **Dalia Lau-Bonilla**[1], William B. Davis, and Anthony G. Garza

Journal of Bacteriology, April 2005, Vol. 187, No. 7, p. 2439-2447.

ABSTRACT

Modification of metabolic pathways to allow for a dormant lifestyle appears to be an important feature for survival of pathogenic bacteria within the host. One regulatory mechanism for persistent *Mycobacterium tuberculosis* infections is the stringent response. Here we analyze the stringent response of a nonpathogenic, saprophytic mycobacterial species, *M. smegmatis*. The use of *M. smegmatis* as a tool for studying the mycobacterial stringent response was demonstrated by measuring the expression of two *M. tuberculosis* genes, *hspX* and *eis*, in *M. smegmatis* in the presence and absence of *relMsm*. The stringent response plays a role in *M. smegmatis* cellular and colony formation that is suggestive of changes in the bacterial cell wall structure.

[1]Dalia Lau-Bonilla performed lipid extractions and TLC analyses.

APPENDIX B

Characterization of a spontaneous hyper-motile *lsr2* mutant of *Mycobacterium smegmatis* derived from a stringent response-deficient parental strain.

Kriti Arora, **Dalia Lau-Bonilla**[1], Danelle C. Whiteford, Eric A. Shelden, Christine M. Davitt, and John L. Dahl

Journal of Bacteriology, June 2008, Vol. 190, No. 12, p. 4291-4300.

ABSTRACT

Mycobacterial species are characterized by the presence of lipid-rich, hydrophobic cell envelopes. These cell envelopes contribute to properties such as roughness of colonies, aggregation of cells in liquid culture without detergent, and biofilm formation. We report here a mutant of *Mycobacterium smegmatis*, called DL1215, which arose spontaneously from a strain deficient for the stringent response (*M. smegmatis* Δrel_{Msm}). DL1215 is not a reversion to a wild-type phenotype, and it shows characteristics unique from both the wild-type and the Δrel_{Msm} parental strains. Compared to these two strains, DL1215 displays a greatly enhanced ability to slide over agar surfaces, and it has greater resistance to phage infection and to the antibiotics kanamycin and isoniazid. DL1215 phenotypic differences are not due to changes in

glycopeptidolipids, polar lipids, apolar lipids, or mycolic acids. Phage-like structures are visible protruding from DL1215 cells while sliding on agarose plates and these structures may represent a novel method of bacterial locomotion. Complementation of the DL1215 mutant with a plasmid containing the *lsr2* gene reverts the strain to the *M. smegmatis* Δrel_{Msm} phenotype. Sequence analysis of the *lsr2* gene of DL1215 revealed a single base pair deletion leading to formation of a truncated protein product.

[1]Dalia Lau-Bonilla provided protocols and assistance with lipid extractions and TLC analyses presented in this paper.