STRESS SURVIVAL IN *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* AND THE ROLE OF HUP IN *MYCOBACTERIUM SMEGMATIS*

By

DANELLE WHITEFORD

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of DANELLE WHITEFORD find it satisfactory and recommend that it be accepted.

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Chair

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STRESS SURVIVAL IN *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS* and THE ROLE OF HUP IN *MYCOBACTERIUM SMEGMATIS*

Abstract

by Danelle Whiteford, Ph.D
Washington State University
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Chair: John L. Dahl

This study characterizes differences in the abilities of two closely related *Mycobacterium* species to survive stress. *M. tuberculosis* and *M. bovis* are both human pathogens, but *M. bovis* is also a major animal pathogen and is more virulent in small animal hosts. In this study it was shown that *M. bovis* was more resistant to the stress of desiccation, low pH, and high heat. Using TLC, this study also demonstrated differences between the cell envelopes of these two species. Finally a comparison of the proteomes made between *M. tuberculosis* and *M. bovis* revealed differences in protein expression levels. One protein of interest that had elevated levels in *M. bovis* was the histone-like protein HupB.

In an effort to elucidate the function of HupB in *Mycobacterium*, the *hupB* homolog (*hup*) was deleted from the chromosome of the non-pathogen *M. smegmatis*. *M.*

smegmatis was chosen for study due to its shorter doubling time and frequent use as a model system for studying its pathogenic counterparts. Initial observation of *M. smegmatis* Δ*hup* revealed a colony morphology that was smoother than the typical rough colony of wild-type *M. smegmatis*. Unlike wild-type *M. smegmatis*, the Δ*hup* strain grew dispersed in liquid cultures, with minimal clumping. Loss of *hup* does not affect long-term viability of *M. smegmatis* but it does leave cells more susceptible to UV light and freeze/thaw treatment. In addition *M. smegmatis* Δ*hup* is four times more susceptible to the action of the front-line antibiotic isoniazid. Although the complete molecular function of Hup has not yet been determined, this work demonstrates that Hup does have a role in the structure of the cell envelope and in the resistance to some environmental stresses.

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Introduction

The Genus *Mycobacterium*

The genus *Mycobacterium* is a member of the family Mycobacteriacae consisting of cells characterized by long slender rods and a unique cell wall structure. The complex nature of the *Mycobacterium* cell wall prohibits designation as either Gram positive or Gram negative. Instead, the waxy cell wall lipids and mycolic acids allow mycobacteria to retain a carbolfuschin stain even after washing with acid alcohol. Because of this mycobacteria are considered "acid-fast." Acid-fast staining is not a unique characteristic of mycobacteria, however as *Nocardi*a and *Rhodococcus* species also have this staining feature (88).

To date over 100 Mycobacterial species have been identified. The growth rates of these species can be relatively fast such as *Mycobacterium smegmatis*, which has a doubling time of 4-6 hours, or relatively slow such as *Mycobacterium tuberculosis*, which has a doubling time of 18-20 hours. Among these various species are a number of clinically important pathogens. *Mycobacterium marinum* primarily causes infections in fish, but is also responsible for what is known as "fish-tank granulomas" in humans (7). *Mycobacterium leprae* is responsible for the historically devastating disease leprosy (13). *Mycobacterium tuberculosis* is the causative agent of the disease tuberculosis (TB), which continues to be responsible for approximately 2 million deaths each year (40).

The research presented in this dissertation involves the examination of three Mycobacterial species: *M. smegmatis*, *M. tuberculosis*, and *M. bovis*.

Mycobacterium smegmatis

M. smegmatis is a soil-dwelling, fast-growing photochromogen, first observed in 1884. It is classified as nonpathogenic; however, like most *Mycobacterium, M. smegmatis* is capable of establishing the rare infection in humans. Reports have identified *M. smegmatis* as the cause of skin and soft tissue infections (71, 110), catheter-related bacteremia (97), pleuropulmonary infections (56, 108), and a fatal disseminated infection in an immune compromised infant (79).

Working with *M. smegmatis* does not require a BSL-3 containment facility, which is required for working with the pathogenic *Mycobacterium* species, *M. tuberculosis* and *M. bovis. M. smegmatis* also has a much shorter doubling time compared to its pathogenic counterparts. Instead of the 3-4 weeks that it takes for a *M. tuberculosis* colony to appear on solid media, a *M. smegmatis* colony will appear in 3-4 days. For these reasons, *M. smegmatis* has become a popular model system for studying gene regulation and physiology of pathogenic *Mycobacterium* (25, 31, 47, 77, 87, 112). Expression of *Mycobacterium* genes in *E. coli* is often met with limited success, in part due to the high GC content of mycobacterial DNA (57). With a typical GC content of around 65-70%, many common mycobacterial codons are considered rare in *E. coli* (57). This can lead to truncated proteins when *E. coli* is used as an expression host. Therefore, in place of *E. coli*, *M. smegmatis* can be used as an expression system for genes from pathogenic *Mycobacterium* species (122).

Although *M. smegmatis* lacks the pathogenic properties of *M. tuberculosis*, it contains homologs to many *M. tuberculosis* virulence genes (86) as well as sigma factors

and two-component systems (107). This, along with the conservation of housekeeping genes, creates a usefully surrogate host for studying the expression of genes from pathogenic *Mycobacterium*. While *M. smegmatis* may not be suitable for studying some aspects of *Mycobacterium* virulence it can be used to study important physiological conditions such as bacterial dormancy. A technique that has long been used for stimulating dormancy in *M. tuberculosis in vitro* (111) has proven effective in generating a similar dormant physiology in *M. smegmatis* (31).

There are of course limitations to using *M. smegmatis* as a surrogate host. The *M. smegmatis* genome is 1.7 times larger than that of *M. tuberculosis* (86). As a result of this there could be situations of gene redundancy in M. *smegmatis* that are not present in *M. tuberculosis*. For example, the gene *lsr2* appears to be an essential gene in *M. tuberculosis* (18, 93), but has been successfully deleted from the *M. smegmatis* chromosome (4, 14). Ultimately the usefulness of *M. smegmatis* as a model system and surrogate host seems to outweigh the limitations. This is especially evident in a recent report identified a promising new class of anti-tuberculosis drugs called diarylquinolines, by screening antibiotic activity against *M. smegmatis* (53).

M. tuberculosis **and** ***M. bovis***

Robert Koch first isolated *M. tuberculosis* as the causative agent of tuberculosis in 1882. Not long after Koch's discover,y Theobald Smith noticed differences in the bacteria isolated from infected humans and infected cows (81). Concluding that there

were two separate species, a human tubercle bacillus and a bovine tubercle bacillus, Koch proclaimed that the bovine tubercle was unable to infect humans (89). This statement proved incorrect. While the human tubercle bacillus was in fact unable to infect cows, the bovine tubercle bacillus did cause disease in humans. It would take years for this controversy to be settled and for the removal of infected cows to become the established practice (89). By today's molecular standards, *M. bovis* and *M. tuberculosis* would likely be considered two strains of the same species, perhaps with *M. bovis* as a subspecies of *M. tuberculosis*. (89). However, this practice has not been adopted, likely due to resistance in the medical and verterinary communities.

Both *M. tuberculosis* and *M. bovis* are members of the tuberculosis complex of *Mycobacterium* pathogens. Other members of this complex include *M. africanum*, *M. canettii*, *M. pinnipedii*, *M. caprae* and *M. microti* (98). All organisms in the *M. tuberculosis* complex are capable of causing the disease known as tuberculosis in humans. Additionally, some of these organisms share a startling similarity. The genomes of *M. tuberculosis* and *M. bovis* have greater than 99.95% sequence identity (19, 38). This is a sequence divergence of less than 0.05%. To put this in perspective, two strains of *E. coli* have a sequence divergence of 1.6% (98). Due to this similarity, differentiating *M. bovis* from *M. tuberculosis* can be difficult. Historically, speciation has been conducted by biochemical tests. For instance, in contrast to *M. tuberculosis*, *M. bovis* is negative for nitrate reduction and niacin accumulation (123). *M. bovis* is also considered insensitive to the antibiotic pyrazinamide (PZA) (123). This lack of sensitivity is due to a single nucleotide polymorphism in the *pncA* gene, the product of which is responsible for activation of the PZA prodrug (121).

Recent genomic comparisons between *M. bovis* and *M. tuberculosis* have revealed that *M. bovis* contains no unique genes when compared to *M. tuberculosis* (41). *M. tuberculosis* does however contain nine regions of DNA, called regions of deletion (RD4-RD10, RD13-RD14), that are missing from the *M. bovis* chromosome (Fig. 1) (43). Despite the loss of genetic information from *M. bovis*, this pathogen has a wider host tropism compared to *M. tuberculosis* (84). Both species are capable of causing disease in humans. However, while *M. tuberculosis* infections are limited to humans, *M. bovis* infections appear in cattle, deer and other wild and domestic animals (46, 20). *M. bovis* also demonstrates greater virulence in mice, rabbits and guinea pigs when compared to *M. tuberculosis* (34). So what causes such differences in host tropism and virulence in two pathogens that have such a high level of sequence identity? One possibility is that differential gene expression plays a large role (41, 84). Two recent studies have used microarray technology to identify major differences in the transcriptomes of these two organisms (41, 84). Golby *et al.* identified a set of 92 genes that were differentially regulated in steady state cultures (41). Rehren *et al*. identified even more differentially regulated genes, with 278 genes showing different expression levels in early logarithmic cultures (84). Differentially regulated genes included transcriptional regulators such as *whiB6*, a gene found with higher expression in *M. bovis* compared to *M. tuberculosis* in both studies (41, 84). *whiB6* is a major stress response transcriptional regulator found upregulated in response to heat and other stresses (39). Also found to have higher expression in *M. bovis* was the major secreted antigen MPB70 (41, 84). Other differentially expressed genes identified were involved in lipid metabolism and protein secretion (41, 84).

Although differential gene expression may be responsible for differing behavior in animal hosts, *M. tuberculosis* and *M. bovis* generate identical symptoms upon infection of humans. In fact most cases of *M. bovis* are likely attributed to *M. tuberculosis* since infection is usually diagnosed by the symptoms, and the isolation of acid-fast organisms, rather than actual species identification (105). Since Robert Koch identified *M. tuberculosis* as the causative agent of TB, there have been extensive developments into the treatment and prevention of this disease. Unfortunately TB continues to be a world health problem. It is estimated that one third of the world's population is infected with a member of the *M. tuberculosis* complex. Of those infected only around 10% will actually develop TB. A vaccine does exist for tuberculosis. This vaccine is an attenuated strain of *M. bovis* called the bacillus of Calmette and Guérin (BCG) strain. This strain was created in 1921 by passing a virulent strain of *M. bovis* 230 times until the organism was considered safe for human use (11). The attenuation of *M. bovis* BCG is attributed to the loss of a 10-Kb region of deletion (RD1), which contains the gene *esat6* (11). The product of this gene is a secreted protein that acts as an important stimulator of the immune response upon early infection (100). Unfortunately the efficacy of this vaccine is extremely variable, particularly with respect to pulmonary TB. It does appear to offer some protection in children against disseminated forms of tuberculosis but is not considered protective in adults (11).

M. tuberculosis is an air borne pathogen that is spread by aerosolized droplets when an infected person coughs. Upon inhalation, the bacteria enter the lungs and are phagocytized by alveolar macrophages. This triggers a proinflammatory response that recruits mononuclear cells to the infected macrophage. Together these cells form the

granuloma, an aggregation of giant cells surrounding the infected macrophage (91). The granuloma serves to prevent bacterial dissemination but also acts to protect *M. tuberculosis* cells from antimicrobial mechanisms of the host, as the bacilli essentially hide out inside the macrophage (90). Within a granuloma *M. tuberculosis* can enter a latent state (42). In most cases the host will never experience any symptoms. However, if the host's immune system becomes compromised the granuloma containment can fail and the latent bacilli can enter the airways becoming infectious (90). This activation will lead to the symptoms associated with disease such as a wet cough, fever, malaise and weight loss (78). *M. bovis* can also enter a host through inhalation and establish infection in the manner described above. However, today *M. bovis* is more commonly passed to humans by ingestion of non-pasteurized cow's milk (5). Upon ingestion the organism enters the stomach where its ability to withstand a low pH allows it to survive acidic digestion and invade the intestinal wall (63). As the bacteria enter the blood or the lymphatic system they spread to sites all over the human body and establish foci of infections (63). Phagocytosis by macrophage can lead to the formation of a granuloma as previously described. The most common site of granuloma formation due to an extrapulmonary infection is the lymph nodes (5, 52).

Within the granuloma it is believed *M. tuberculosis* and *M. bovis* face a harsh environment of low oxygen and low nutrients (67). As airborne pathogens, both organisms also encounter desiccation, UV exposure and lower than optimal temperatures. A thorough understanding of the mechanisms used by the bacteria to survive stress is a major area of *Mycobacterium* research as it could be useful in the fight against these pathogens.

The Bacterial Stress Responses

The life of a bacterial cell can be hazardous as it is exposed to various stressful conditions. From desiccation to low nutrients, environmental stresses can generate complex physiological responses from bacterial cells attempting to survive. Studies aimed at understanding bacterial responses to stress have focused on both the physiological changes that cells undergo when stressed, as well as the regulation of genes controlled by a stress response (2, 23, 29, 54, 61, 65).

Specific alterations in an environment can generate very specific cellular responses. For example, heat shock in *Escherichia coli* leads to a temporary increase in the alternative sigma factor, σ^{32} (120). This sigma factor, along with σ^{24}, is responsible for an increase in production of heat shock proteins, which can aid in folding and repair of heat-damaged proteins. In mycobacteria two forms of regulation control the expression of heat inducible genes: release of repression by the proteins HspR or HrcA, and induction by three alternative sigma factors (67, 101). Through these control mechanisms heat shock leads to the induction of chaperones (heat shock proteins conserved across bacterial species) such as GroEL, DnaK, and GroES. Interestingly heat shock proteins are also induced upon Mycobacterial infection and uptake by macrophages (59, 82). Their induction might aide in the organism's ability to survive the stresses produced by the macrophage (59, 82). Chaperones are major antigens that are involved in stimulating the immune response of the host (118). While induction of proteins such as DnaK may be beneficial for the microbe in surviving stress, stimulating

an immune response could also be detrimental to infectious bacilli. Over-expression of DnaK was shown to decrease the ability of *M. tuberculosis* to persist in a chronic infection (101). Chaperones may be a major part of the heat shock response, but they are not the only cellular reaction to increase in temperature. Heat shock also causes genome wide changes in the transcriptional profile of mycobacteria (102).

Cold shock induces a different cellular response in bacteria. Since low temperatures can have a negative effect on the initiation of translation, a series of cold shock proteins are produced (92). These proteins can prevent the formation of secondary structures in the RNA and allow for translation initiation (92, 96). One well-studied cold-shock inducible protein in *E. coli* is H-NS, a nucleoid-associated protein (114). H-NS is believed to be involved in the compaction of the chromosome and in influencing negative supercoiling (26, 73, 106). When the gene for H-NS was inactivated in *E. coli* the cells showed an increased sensitivity to cold suggesting that the topology of the chromosome can be important in surviving a decrease in temperature (30, 92). In *M. smegmatis* cold shock is known to cause the induction of another DNA binding protein called CipMa (96), also known as HupB. CipMa is a major cold-inducible protein in *M. smegmatis*, with induction occurring 2-3 hours after a decrease in temperature to 10°C and leading to a total of 10-12-fold induction (96). Given the early induction of CipMA, Shires *et al.* postulated that the protein could have a role in controlling the expression of proteins necessary for *M. smegmatis* to grow at low temperatures (96).

Exposure to UV light is another common stress that bacteria face in the environment. This type of stress can lead to DNA damage such as cyclobutane pyrmidine dimers (CPDs) (115). In the event of DNA damage bacteria can undergo initiation of a

number of repair pathways, one of which, called the SOS response, constitutes a major stress response for bacterial systems (45). The SOS response is characterized by the LexA/RecA regulon. In this system, LexA acts as a repressor for the expression of genes involved in DNA damage repair. LexA binds to a consensus sequence called the SOS box, which is located upstream of SOS inducible genes (95). When damage occurs, LexA is cleaved by a RecA stimulated reaction and is unable to bind to the SOS box efficiently, causing an increase in expression of the LexA regulated genes (45). Mycobacteria have a similar SOS response to that of the well-studied *E. coli* system (28 69). However, microarray analysis has indicated another major regulatory system in *M. tuberculosis* that is independent of LexA/RecA (83). Genes involved in nucleotide excision repair and recombination were among those induced in a RecA-independent manner (83). Davis *et al.* identified a second promoter region upstream of the *M. tuberculosis recA* gene, which was induced upon DNA damage but independent of LexA (27). This promoter matched a promoter motif found upstream of the majority of DNA damage inducible genes in *M. tuberculosis,* including those involved in recombination repair and nucleotide excision repair (37). This discovery led to the conclusion that although some DNA damage inducible genes are regulated similarly to *E. coli*, the majority of them are controlled independent of LexA repression (37).

Examination of specific bacterial stress responses such as starvation, UV exposure, and cold, has pointed to the involvement of a family of proteins called "histone-like" proteins in stress adaptation and survival (15, 64, 96, 109).

Histone-Like Proteins

In eukaryotic cells DNA is coiled around histone octamers to form nucleosome core particles. This octamer consists of two each of histones H2A, H2B, H3, and H4. This level of organized compaction of DNA does not occur in prokaryotic cells (104). However, DNA must still be compacted in what was once believed to be an unordered arrangement of randomly coiled DNA, but now appears to be a regulated manner necessary for efficient gene expression (104). One class of proteins called histone-like proteins may have specific roles in altering the structure of the bacterial chromosome (Fig. 2). Histone-like proteins are generally small basic, DNA binding proteins that are found in high abundance in the bacterial cell (104).

In *E. coli* two of the most abundant histone-like proteins are HU and IHF (integration host factor). Both proteins are members of the DNABII family of DNA binding proteins, a family of proteins that is highly conserved across prokaryotic species (103). Along with other DNA binding proteins, HU and IHF are involved in compaction of the *E. coli* chromosome (1, 8). In addition DNA bending by HU or IHF controls transcriptional regulation for over 120 *E. coli* genes (3). By regulating DNA topology, histone-like proteins can directly affect the activity of transcription factors (16, 48, 75). For example, IHF can act to loop DNA bringing regulatory proteins bound at upstream sites into contact with RNA polymerase bound downstream at the gene promoter site (16, 48). In *M. smegmatis* a histone-like protein called LSR2 has been shown to be regulate transcription of an operon that confers multi-drug tolerance (18). Along with regulating

gene expression by controlling DNA supercoiling, histone-like proteins have also been shown to have a role in initiation of DNA replication and cell division (73, 116, 119)

Underscoring the importance of histone-like proteins in bacterial cells, a number of different phenotypes have been observed when the level of these proteins has been altered. HU forms a heterodimer consisting of two low molecular weight proteins HUα and HUβ. Inactivation of both HUα and HUβ leads to loss of long-term viability (15), an increase in the sensitivity to cold (109), and an increase in UV sensitivity (64). Loss of HU also negatively affects *recA* dependent recombinational DNA repair and induction of the SOS pathway upon exposure to UV (64, 68). The overproduction of one of the HU subunits leads to an increase in colony mucoidy and filamentous cells (74). Expression of another *E. coli* histone-like protein H-NS on a high copy number plasmid is lethal to the cell, while expression on a low copy number plasmid again leads to the formation of filamentous cells (94). Taken together, these studies suggest a complex involvement of histone-like proteins in the physiology and gene regulation of prokaryotes.

Mycobacterium Histone Like Protein HupB

The genus *Mycobacterium* has a single homolog to the *E. coli* HU protein, called HupB (also known as Hup, Hlp, LBP, and MDBPI). HupB is a 265-amino acid, 21-kDa, protein with highly basic isoelectric point of 12.5. Mycobacterial HupB shares sequence similarity to both prokaryotic histone-like proteins, through its N-terminal domain, and the eukaryotic histone HI, through is C-terminal domain. Similarity to histone HI is primarily due to a high concentration of the amino acids alanine, lysine, and proline. The

HupB protein is post-translationally modified by methylation of lysine residues (99) as is seen with eukaryotic histones (17).

HupB is abundant in mycobacterial cells making up 7-10% of the total cellular protein (36). It has been found to bind DNA nonspecifically (36, 80) but appears to prefer GC rich binding sites (36). Both the C-terminal and N-terminal domains of HupB are involved in binding of DNA (70). Binding of HupB to DNA *in vitro* inhibits DNA synthesis, transcription, and translation (36). These results highlight the potential importance of mycobacterial histone-like proteins in cellular function. In addition to being localized in the cytosol (70, 80), HupB has also been localized to the cell wall of *M. leprae* (99) and *M. tuberculosis* (117). In *M. tuberculosis*, HupB was recently shown to interact with Ag85 to transfer mycolic acids to sugar components in the cell envelope (51). This same study linked HupB with the growth stage dependent down-regulation of components of the cell envelope (51). In *M. leprae* HupB on the cell surface binds laminin aiding in the attachment of the mycobacterial cells to alveolar epithelial cells and macrophages (99). HupB could therefore have a role in host tropism. Further evidence to support the role of HupB in cell-to-cell interactions was found through the study of a HupB knockdown in *M. bovis* BCG (62). The decreased expression of HupB impaired aggregation between *M. bovis* cells (62).

As was seen with *E. coli* HU proteins, mycobacterial HupB appears to have some role in stress response. HupB has been identified as upregulated in response to cold-shock (96), anaerobiosis (60), and low iron (117). Microarray analysis also showed an upregulation of HupB by the stringent response, a stress response activated by starvation

(24). At present the potential role of this protein in survival during stress conditions is not known.

hupB is predicted to be an essential gene in *M. tuberculosis* (93). In *M. smegmatis* however, a *hupB* knockout has been generated (60). This mutant demonstrates no difference in growth rate or loss of viability after two weeks of growth. This suggests some redundancy in the function of the *M. smegmatis* HupB. The *hupB* knockout does appear unable to recover after exposure to cold-shock (96).

The Mycobacterial Cell Wall

As mentioned above, the genus *Mycobacterium* is characterized by an unusually complex cell wall that is extremely lipid rich. Lipids account for up to 60% of the dry weight of the mycobacterial cell wall and around 250 genes are involved in lipid metabolism (19, 44). This is compared to the approximately 50 genes found to be responsible for lipid metabolism in *E. coli* (19). The lipid rich nature of the mycobacterial cell wall is responsible for many of properties that make *Mycobacterium* such deadly pathogens, such as resistance to antibiotics and environmental stresses. The mycobacterial cell wall consists of two segments (Fig. 3). The inner segment is just outside of the plasma membrane. It is composed of peptidoglycan, to which a polysaccharide polymer called arabinogalactan is covalently attached (10). Forming a covalent linkage with arabinogalactan are the mycolic acids. Together the peptidoglycan-arabinogalactan-mycolic acids make up the inner core of the mycobacterial cell wall (Fig. 3) (10). The outer segment of the cell wall is composed of free lipids, loosely associated

with, but not attached to, the inner core (Fig. 3) (10). Surrounding the whole cell wall structure is a polysaccharide and protein capsule (32).

The structure of the plasma membrane and peptidoglycan of *Mycobacterium* is not that dissimilar to that of other bacterial species. However, there are a few important characteristics that should be mentioned. The plasma membrane, for instance, is home to the carotenoids, believed to be involved in photoprotection (9). It is the carotenoids that are responsible for the yellow pigmentation of photo-chromogenic species such as *M. smegmatis*. In addition, attached to the plasma membrane are the phosphatidylinositol mannosides (PIMs). These form the lipid base for lipoarabinomannan (LAM) (9). LAM is a major cell wall species that extends through the cell wall to the outer surface where it can bind Toll-like receptors and initiate signaling events in host cells (10). Mycobacterial peptidoglycan has two distinct differences to that of other bacterial species. First, while the peptidoglycan of *E. coli* consists of alternating N-acetylglucosamine cross-linked to N-acetylmuramic acid (NAG), the mycobacterial peptidoglycan uses N-glycollylmuramic acid (NGM) (32). Second, additional cross-links exist between alternating units of NAG and NGM (10).

Covalently attached to the peptidoglycan is the major cell wall polysaccharide, arabinogalactan. The attachment occurs through the galactan region of the polysaccharide, which forms a bridge with some of the N-glycollymuramic acid species found in the peptidoglycan (10). Attached to the arabinogalactan via an ester linkage are the mycolic acids, long chain branched α-alkyl-β-hydroxy fatty acids composed of 60-90 carbon atoms (44 58). Mycolic acids form the inner leaflet of an asymmetrical lipid bilayer that is primarily responsible for the resistance of *Mycobacterium* species to many

common antibiotics. Jackson *et al*. found that a decrease in the number of mycolic acid species in the cell wall led to an increase in permeability for both hydrophobic and hydrophilic molecules (49). Two front-line antimycobacterial drugs, ethambutol and isoniazid, target arabinogalactan and mycolic acid synthesis, respectively. The importance of the cell wall as a barrier against the environment has attracted a lot of interest as scientists search for new antibiotics to fight against mycobacteria.

Forming the outer leaflet of the lipid bilayer is a number of free lipids that are intercalated within the arabinogalactan-mycolic acids complexes. These free lipids include phthiocerol dimycocerosate (PDIM), phenolic glycolipids (PGLs), and trehalose dimycolate (cord factor). As many of the free lipids are surface exposed, they play major roles in the activation of the host immune response and other host-pathogen interactions. For example, mutant strains of *M. tuberculosis* that cannot synthesize PDIMs are unable to replicate inside mouse lungs, but can grow in liver or spleen (22). Strains deficient in PDIMs also have increased permeability and are more sensitive to detergents (22). PGLs are strong antigens that are found in *M. bovis* but absent from most *M. tuberculosis* strains due to a frameshift mutation in *pks15/1*, a gene involved in PGL synthesis (21). Finally, trehalose dimycolate, commonly called cord factor, is toxic to mice when administered repeatedly in small doses (10). Toxicity is caused by the ability of cord factor to diffuse into host cell membranes, namely mitochondrial membranes, and disrupt functions (50, 113). Cord factor can also induce the formation of lung granulomas in mice (6, 55). Together these studies emphasize the importance of the free lipids both in maintaining the structure and impermeability of the cell wall and in interactions with host cells.

The lipid bilayer formed by the mycolic acids and free lipids of mycobacteria has incited comparisons to the outer lipid membrane found in Gram-negative bacteria; although *M. tuberculosis* cells are 100 times less permeable than *E. coli* (32). Gram-negative bacteria have adapted outer membrane proteins, called porins, which allow passage of small hydrophilic nutrients through the outer membrane. Mycobacterial cell walls have also been shown to have similar porins extending through the lipid bilayer. One of the major porins identified in *M. smegmatis*, called MspA, is part of a family of four outer membrane proteins. Structurally, MspA forms a single pore through the lipid bilayer and is over three times longer than the porins found in Gram-negative bacteria (35). When the *M. smegmatis* MspA was expressed in *M. tuberculosis* it led to a significant increase in permeability and susceptibility to antibiotics such as isoniazid and ethambutol (66). *M. bovis* and *M. tuberculosis* also have genes predicted to be responsible for porins, but these proteins have not been well characterized (33). One *M. tuberculosis* porin that has been studied, $OpmA_{TB}$ is still debated for its role as a porin (33). $OmpA_{TB}$ is too small to extend through the length of the mycobacterial cell lipids (33) and deletion of $ompA_{TB}$ led to only a slight decrease in permeability (85).

The last component of the mycobacterial cell wall is the capsule composed of polysaccharides and proteins. The capsule has only recently been characterized as it is shed when cells are grown with a detergent and in a shaking incubator, which is typically how *Mycobacterium* are cultured (32). It was originally thought that the capsule was also made of lipids, but Ortalo-Magne *et al*. isolated the capsule material and determined that it consisted of only 6% lipid and was primarily protein and polysaccharide (72). The

capsule represents the final barrier between the environment and the mycobacterial cell wall.

Goals of this dissertation

The research outlined in this dissertation can be divided into two projects:

1) The work described in Chapter 2 examines the differential survival of two closely related species of *Mycobacterium* when exposed to environment stress. This work suggested at the involvement of HupB in a stress response, which led to the second project, discussed in Chapter 3.
2) The work described in Chapter 3 examines the involvement of the protein HupB in stress resistance by studying a *M. smegmatis* HupB mutant and its ability to withstand various stressors including antibiotic exposure.

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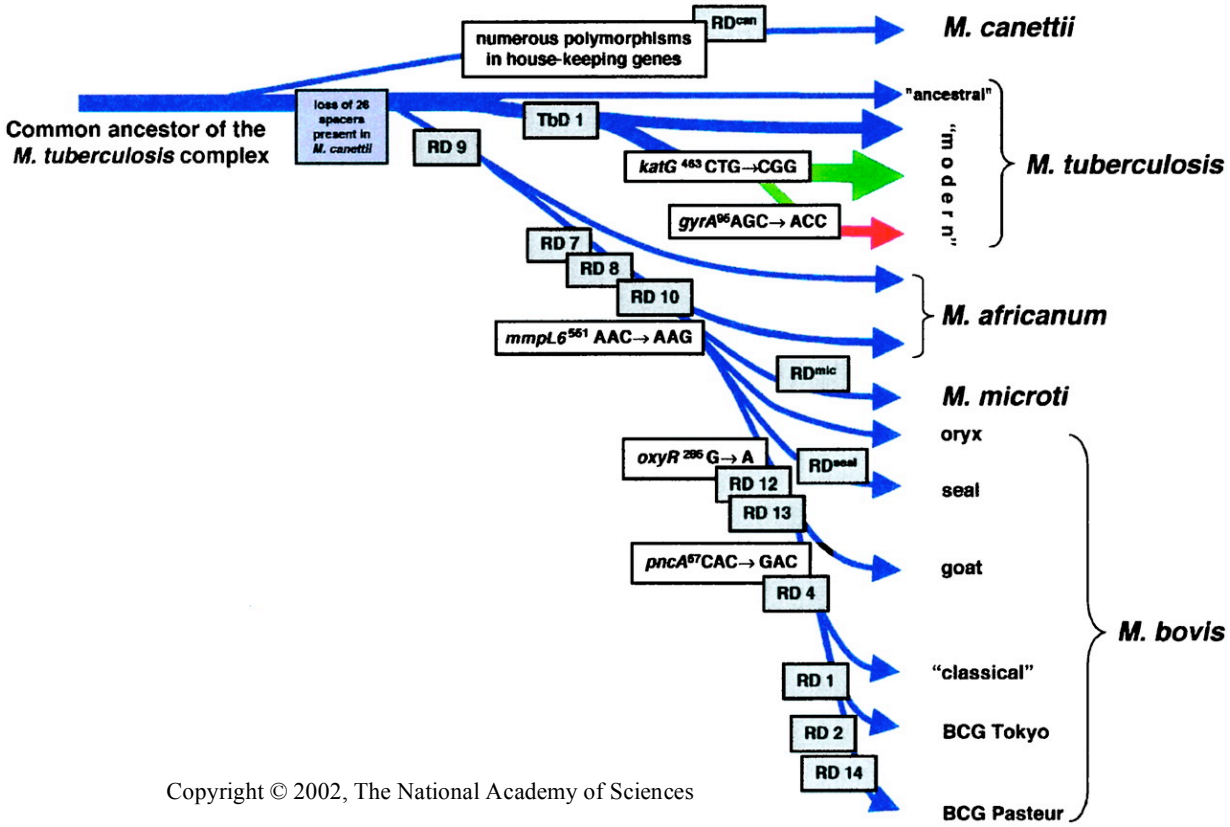

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Figure 1: Predicted scheme for the evolution of the *M. tuberculosis* complex (12). *M. tuberculosis* and *M. bovis* likely evolved from a common ancestor. Gray boxes indicate regions of deletion (RD), sections of DNA that have been lost as the organisms evolved. Some important genetic mutations are also indicated.

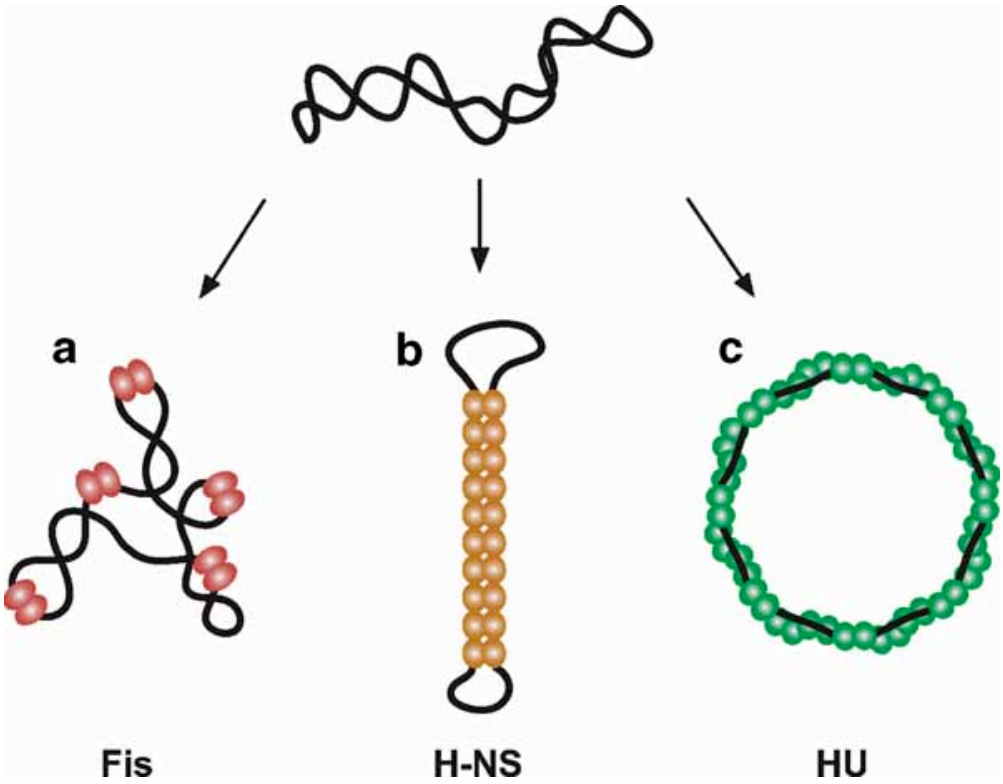

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Figure 2: Histone like proteins can affect the conformation of supercoiled plasmid DNA (104). **(**A) Binding of Fis creates branched DNA. (B) H-NS binding causes DNA to form "dumbbell-shaped" structure. (C) High concentrations of HU open up the DNA to form a ring like structure.

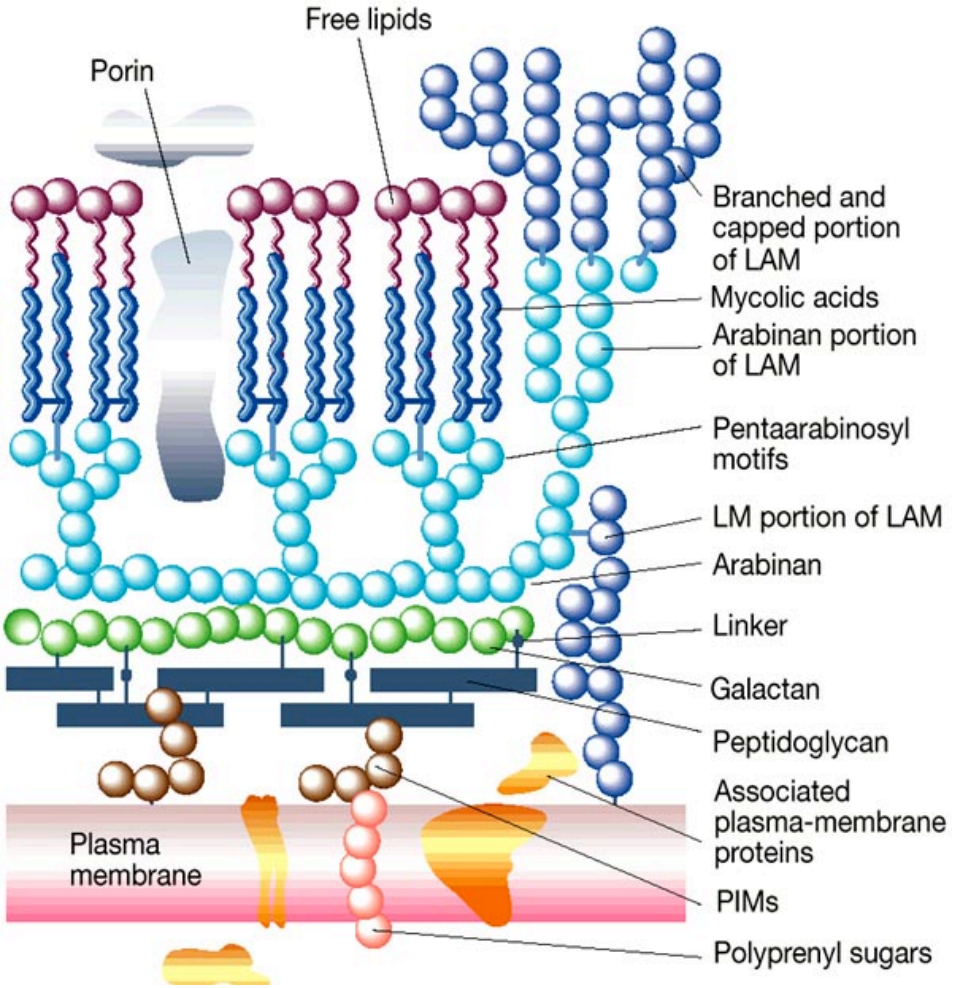

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Figure 3. Mycobacterial Cell Envelope (76). The mycobacterial cell wall is highly complex and lipid rich. Just outside of the plasma membrane is the peptidoglycan, to which a polysaccharide polymer called arabinogalactan is covalently attached (10). The outer segment of the cell wall is composed of free lipids, loosely associated with, but not attached to, the inner core (10). Surrounding the whole cell wall structure is a polysaccharide and protein capsule (not shown on figure). LAM=lipoarabinomannans; LM=lipomannans; PIM=phosphatidylinositol mannosides.

CHAPTER TWO

Mycobacterium tuberculosis and *Mycobacterium bovis* differ in stress resistance and protein expression

Danelle C. Whiteford,[1] Dalia Lau-Bonilla,[1] Kriti Arora,[1] Lisa J. Washburn,[2] William Siems,[2] Christine M. Davitt,[3] and John L. Dahl[4*]

School of Molecular Biosciences, Washington State University, Pullman, Washington 99164,[1] Mass-Spectrometry Laboratory, Department of Chemistry, Washington State University, Pullman, Washington 99164,[2] and Electron Microscopy and Imaging Center, College of Sciences, Washington State University, Pullman, Washington 99164,[3] Department of Biology, University of Minnesota Duluth, Duluth, Minnesota 55512[4]

*Corresponding author. Mailing address: University of Minnesota Duluth, School of Biology, 207 SSB, 1035 Kirby Dr, Duluth, MN 55812. Phone: (218) 726-7264. E-mail: jldahl@d.umn.edu.

ABSTRACT

Despite having genomes that are >99.95% identical, *Mycobacterium tuberculosis* and *Mycobacterium bovis* exhibit very different host ranges. *M. tuberculosis* is the causative agent of human tuberculosis while *M. bovis* has zoonotic potential and is known to infect a variety of domestic and wild animals. When the two mycobacterial species are directly compared *in vitro* a number of different properties are observed including cell and colony morphologies, growth rates, cell envelope compositions, proteomes, and resistance to stresses of low pH, heat, and desiccation. *M. bovis* appears to be more resistant to the stresses tested compared to *M. tuberculosis* including a 1.5-log increase in survival after acid shock, 5-log increase in survival after desiccation, and a 3-log increase in survival after exposure to heat. A comparison of the proteomes of the two species revealed variations in protein expression levels. One distinguishing feature of the two proteomes is that, compared to *M. tuberculosis*, *M. bovis* has elevated levels of the histone-like protein HupB.

INTRODUCTION

The toll of mycobacterial infections upon human society has diminished little in the past century. *Mycobacterium tuberculosis* is still the leading cause of death globally due to a single infectious agent. Upwards to a third of the world's population is infected with *M. tuberculosis* leading to 2-3 million deaths annually (19). The role of *Mycobacterium bovis* is likewise a significant threat in developing countries (3). Eradication efforts for *M. bovis* are complicated by the existence of wildlife reservoirs including badgers, buffalo, antelope, possums, and deer (10, 35, 52).

2007 marked the 125th anniversary of Koch's discovery of the tubercle bacillus, originally believed to be responsible for both human and bovine tuberculosis infections. However, Koch revised his assessment when he was unable to establish disease in cattle by injecting tubercle bacilli from humans. In 1898 Smith recognized differences between tubercle bacilli that originated in humans or in cattle (46). However, for 60 years following this discovery *M. bovis* was regarded as a subspecies of *M. tuberculosis*. Not until 1957 did Reed treat the bovine bacillus as a separate species (41), and it wasn't until 1970 that the name *M. bovis* was proposed and adopted (24). In 1984 Wayne stated that based upon their high degree of genetic relatedness, *M. tuberculosis* and *M. bovis* would not be considered separate species by most current standards. However, health care and veterinary professionals are opposed to another nomenclature change and bacterial taxonomists are sensitive to this issue (51).

Recently the genomes of two strains of *M. tuberculosis* (H37Rv and CDC1551) (9, 16) and of one strain of *M. bovis* (AF2122/97) (17) have become available and annotations are accessible on the world-wide web (http://genolist.pasteur.fr/TubercuList/)

(http://genolist.pasteur.fr/BoviList/). Comparisons of these sequences reveal that the genome of *M. bovis* is >99.95% identical to that of *M. tuberculosis* (17). *M. bovis* does not have any genes that are not present in the *M. tuberculosis* complex and instead has nine deletions (ranging in size from 1-12 kb) compared to the *M. tuberculosis* strain H37Rv. Therefore, *M. bovis* is likely to have evolved from a common ancestor with *M. tuberculosis* and is not a direct ancestor of *M. tuberculosis* as was originally hypothesized (14). Despite the extensive similarities between the genomes of these two species, they appear to have evolved to survive in a different range of hosts. While infections of *M. tuberculosis* are primarily limited to humans, *M. bovis* infections occur readily in humans, cows, badgers, deer and many more wild and domesticated animals.

Clues to understanding the abilities of these two organisms to infect different hosts might be revealed by recent microarray comparisons of the transcriptomes of mid-log grown *M. bovis* and *M. tuberculosis* cells (42). These experiments have shown that as much as 6% of the total genome is differentially expressed between these two species. *M. bovis* had a larger number of differentially expressed genes for lipid metabolism and regulatory proteins while *M. tuberculosis* had a greater number of differentially expressed insertion sequences. Whereas this microarray study may shed some light on different mechanisms of mycobacterial pathogenicity, it is also beneficial to compare *M. bovis* and *M. tuberculosis* for differences in *in vitro* physiology. Some reports do exist, such as the comparison of the secretion profiles of virulent *M. tuberculosis* and the vaccine strain *M. bovis* BCG (23, 33). In this study we provide a direct comparison between virulent strains of *M. bovis* and *M. tuberculosis* for environmental stress resistance and for a variety of characteristics including cell appearance and composition. Our goal was to

identify any phenotypic differences between these two mycobacterial pathogens. The experiments reported here support the argument that while having very similar genomes, differences in gene regulation in *M. tuberculosis* and *M. bovis* likely result in different *in vitro* phenotypes.

MATERIALS AND METHODS

Growth of bacterial cultures. *M. tuberculosis* strain H37Rv (ATCC 27294) was obtained from Dr. Clifton Barry, III, NIH. *M. bovis* strain 95-1315 was obtained from an infected white-tail deer and was provided by the USDA in Ames, Iowa. This strain displays typical *M. bovis* traits (36) in that it does not produce niacin or reduce nitrate, its growth is inhibited by thiophene-2-carboxylic acid hydrazide, and it is resistant to pyrazinamide. Liquid cultures were grown in either 7H9 liquid medium supplemented with albumin-dextrose-catalase (ADC; Difco), 0.02% glycerol, and 0.05% Tween-80 or Sauton's with 0.05% Tween-80 using a roller-bottle apparatus. Cultures were serially diluted onto 7H11 agar supplemented with ADC and oleic acid (OADC; Difco) and 0.05% glycerol. Colony-forming units (cfus) were examined after 4-5 weeks growth at 37^{o}C.

Microscopy. Cells grown to mid-log phase (OD_{600} 1.0) in 7H9-ADC-Tween were visualized. Scanning electron microscopy (SEM) was performed as previously described (11, 12).

***In vitro* stress tolerance.** Acid tolerance. Sensitivity of the mycobacteria to low pH was determined similarly to a method previously described (37). Briefly, cells were grown in Sauton's medium with 0.05% Tween-80 adjusted to a pH of 6.8. At early log phase (OD_{600} 0.015), cells were resuspended in one-ml aliquots of either fresh Sauton's (pH 6.8) or Sauton's medium with a pH adjusted to a value of 2.0-6.0. Serial dilutions

were preformed on the pH 6.8 sample to determine the colony forming units (cfus) ml^{-1} at the initial (0 h) time point. Samples were incubated in stationary positions at 37°C for 1 day or 4 days before determining cfus ml^{-1} of each sample.

Desiccation tolerance. Survival of bacteria on dry, solid surfaces was performed as described by Archuleta *et al.* (1). Briefly, cells were grown to late logarithmic phase in Sauton's medium, then washed and resuspended in PBS to OD_{600}=1.0. 100µl-aliquots of cells were dried upon sterile glass coverslips and then placed in the dark. At various time intervals, coverslips were placed in 10-ml of Sauton's in 50-ml conical tubes and vortexed to remove cells from coverslips. Serial dilutions were then performed to determine cfus ml^{-1}.

Heat tolerance. Survival of mycobacteria at 53°C was performed as previously described (49). Cultures were grown to early-log phase (OD_{600} 0.015) in 7H9 and then one-ml aliquots were placed in a 53°C water bath for 7.5, 15, and 30 min before serial dilution to determine cfus ml^{-1}.

Cell envelope analysis. Apolar and polar lipids were extracted and analyzed by thin-layer chromatography (TLC), as previously described (5). The lipids were developed using the following systems: Apolar lipids were developed in one dimension 10 times consecutively using petroleum ether: ethyl acetate (98:2); Polar lipids were developed in one dimension using a mixture of chlorofom:methanol:water (65:25:4). All analyses were performed in triplicate. Representative images are shown.

SDS-PAGE analysis of proteins. Protein lysates of mycobacterial cultures were prepared using cells that were grown in 7H9 medium for 10 days with aeration. Harvested cells were washed thrice with PBS, resuspended in phosphate buffered saline (PBS) with Bacterial Protease Inhibitor Cocktail (Sigma), heat killed at 70^{o}C for 30 min, and then lysed with 0.1-mm glass beads using a FastPrep FP120 device (3 intervals of 45 sec each at setting 6.0). Lysates were then centrifuged at 16,000 x g for 10 min. Whole cells and large cell debris in the pellets were suspended in lysis buffer (0.3% SDS, 200 mM DTT, 28 mM Tris-HCl, 22 mM Tris base), boiled for 10 min, and then subjected to three more intervals of lysing with the FastPrep device. After centrifugation supernatants were saved. All protein fractions were subjected to separation by 12% SDS-PAGE and then stained with Coomassie blue.

Protein identification by peptide mass-fingerprinting. Excised Coomassie-stained bands from polyacrylamide gels were treated with trypsin, and eluted peptides were analyzed by Voyager DE RP MALDI-ToF (Applied Biosystems). Monoisotopic peptide masses between the ranges of 700 to 4000 Daltons were used to search the Mascot database (Matrix Science) to identify proteins.

Western blot analysis of HupB protein. Western blots were performed as previously described (3). Primary antibodies used were for HupB (pANCA Fab 5-3) (8). Polyclonal antibodies to whole-cell lysates of *M. tuberculosis* (E193) (Colorado State University) were used to ensure equivalent amounts of cell lysates were transferred to Protran BA83 nitrocellulose membranes (Whatman).

RESULTS

Comparison of *M. tuberculosis* and *M. bovis* growth dynamics. When grown on 7H11+OADC plates *M. tuberculosis* strain H37Rv colonies have a rough, dry, yellowish appearance (Fig. 1A). These colonies are larger than typical *M. bovis* colonies (Fig. 1A). *M. bovis* colonies are typically smooth, transparent, and whitish in appearance (Fig. 1A). In addition to colony morphologies, we compared cell morphologies of *M. tuberculosis* and *M. bovis* (Fig. 1B and 1C). *M. bovis* cells are known to have a slightly different cell morphology. We used SEM to confirm the appearance of shortened bacilli in *M. bovis* compared to *M. tuberculosis* cells (Fig. 1C and 1B, respectively)

To ensure that the differences reported here in response to stress were not due to differences in the growth rate between these two organisms, growth curves were generated with both 7H9+ADC+Tween+glycerol (Fig. 2A) and Sauton's+Tween (Fig. 2B). In these conditions there was no significant difference between the growth of *M. tuberculosis* and *M. bovis*.

Comparisons of stress tolerances for *M. tuberculosis* and *M. bovis*. Acid shock. Pathogenic mycobacteria face exposure to low pH both inside and outside of the host. *M. tuberculosis* and *M. bovis* are able to survive the acidic environment in host macrophages. Exposure to pH 5.5 causes *M. bovis* and *M. tuberculosis* to differentially express a number of genes that are likely important to the survival of the organism in these harsh conditions (20). To determine whether this altered transcriptional response leads to a detectable physiological difference, *M. bovis* and *M. tuberculosis* were

subjected to various pH levels from pH 2.0 to pH 6.0. Compared to *M. tuberculosis*, *M. bovis* showed increased viabilities after exposure to acidic media. Figure 3 shows *M. bovis* had an almost 50-fold increase in viability over *M. tuberculosis* after a 4-day exposure to media at pH 3.5. A pH value of 4.0 or 4.5 yielded similar results (data not shown).

Desiccation tolerance. Desiccation is another stressful condition that pathogenic mycobacteria may be exposed to during the natural cycle of infection. Survival of desiccation may be necessary when the organism is passed from host to host in aerosolized droplets. When compared for desiccation tolerances, *M. bovis* survived longer on glass surfaces than *M. tuberculosis* (Fig. 4A). Although identical after 1.5 h of desiccation, the viability of *M. bovis* was greater than *M. tuberculosis* by 2.5-log, 3-log, and 5-log differences for 3, 6, and 12 days of desiccation, respectively. No viable *M. tuberculosis* cells were recovered at the 12-day time point.

Heat tolerance. *M. tuberculosis* and *M. bovis* were examined for the ability to withstand high heat of 53°C. At this temperature *M. tuberculosis* cells are readily killed and no viable cells were present after a 15-min exposure to this temperature (Fig. 4B). *M. bovis* cells are also killed at 53°C, but the rate of cell death is slower.

Analysis of cell envelopes. Since *M. bovis* colonies lack the rough appearance of *M. tuberculosis* colonies (Fig. 1), they may have altered cell envelope content. In addition the cell envelope is a major barrier against stress and the environment, so differences seen here could be involved in the differential stress survivals reported earlier. The wet weights of cell pellets from different liquid cultures were equilibrated

before lipids were extracted from cell envelopes. The polar lipid profiles of the mycobacteria show two spots absent from *M. bovis* that are present in the *M. tuberculosis* extract (Fig. 5A; closed arrows). Reciprocally, there are polar-lipid species extracted from *M. bovis* cell envelopes that are absent from *M. tuberculosis* (Fig. 5A; open arrows). Apolar lipid profiles show two spots present in *M. bovis* extract that are diminished in the *M. tuberculosis* extract (Fig. 5B; open arrows) and one spot more prevalent in *M. tuberculosis* extract (closed arrow).

SDS-PAGE and Western blot analysis. Repeated 1-D SDS-PAGE analysis showed the majority of proteins in the two species were identical in terms of band positions and Coomassie-staining intensities. However, there were several protein species that differed in staining intensities (Fig. 6A). One protein band was found to be more prevalent in *M. bovis* than in *M. tuberculosis*. This protein was identified as HupB (Mb3010c; Rv2986c) by peptide-mass fingerprinting (Fig. 6A; open arrow). Other identified protein bands more prevalent in *M. bovis* than in *M. tuberculosis* included the major secreted immunogenic protein MPB70 (Mb2900; Rv2875) and a band containing a mixture of two proteins of unknown function (Mb0222 and Mb3102c; Rv0216 and Rv3075c). Western blot analysis verified the elevated levels of HupB (Fig. 6B; arrow) in *M. bovis*. HupB levels are 4-5 fold higher in *M. bovis* than in *M. tuberculosis*. Antibodies to whole-cell lysates show equivalent loading of protein lysates subjected to Western blot analysis (Fig. 6B; bottom panel).

DISCUSSION

Here we report differences in cell shape and colony morphology between *M. bovis* and *M. tuberculosis*. The colonies shown here (Fig. 1A) are similar to those previously reported in which *M. tuberculosis* shows 'eugonic' (abundant) growth on glycerol containing media and results in raised, ruffled colonies. *M. bovis*, however, displays 'dysgonic' (sparse) growth on glycerol containing media with colonies appearing flat and moist (51). This difference is reportedly due to the glycerol effect on *M. bovis* growth since *M. bovis* has a non-functioning pyruvate kinase that results from a single nucleotide polymorphism (SNP) in the *pykA* gene preventing the organism from using glycerol as a carbon source (26). Complementation of *M. bovis* with the *pykA* gene restores the growth of *M. bovis* to the larger, ruffled colonies associated with *M. tuberculosis*.

Compared to the human tubercle bacillus, *M. bovis* has enhanced viability when subjected to environmental stresses despite having greater than 99.95% sequence identity. It is possible that *M. tuberculosis* and *M. bovis* demonstrate different host ranges because of different bacterial viabilities within the hosts. Alternatively, the two species may survive differently in the extra-cellular environment. Of greatest interest would be differences that allowed cells to be more resistant to stresses like those encountered in the host (low pH) or encountered outside the host (desiccation and heat).

Enhanced *M. bovis* survival at a low pH (Fig. 3) suggests it may have an advantage over *M. tuberculosis* in a naturally acidic environment such as would be encountered when the organism is ingested. This might explain the number of

extrapulmonary infections caused when humans have consumed nonpasteurized milk from *M. bovis* infected cows (2). In these cases *M. bovis* is able to survive the acidity of the stomach and pass through to the intestines where the bacilli can invade the intestinal cell wall. Analysis of gene expression in response to pH indicated significant differences in the responses of *M. bovis* and *M. tuberculosis* (20). For example, seven genes involved in proton export were down-regulated in *M. tuberculosis* but not in *M. bovis* (20). Proton export could aid in maintaining the pH in the cytoplasm, so this difference could play some role in the difference in viabilities we have observed here.

The enhanced desiccation tolerance of *M. bovis* (Fig. 4A) may be due to altered cell surface properties. A published report of transcriptional comparisons show *M. bovis* has higher expression of fatty-acid-coA ligase and polyketide synthase; two enzymes involved in phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL) biosynthesis (42). Genomic comparisons between *M. tuberculosis* and *M. bovis* suggest many differences in the two species may occur on the cell surface or in secreted substances (17). This seems especially true in the PE-PGRS and PPE family members, which are suspected of being surface-exposed and show large blocks of variation between *M. tuberculosis* and *M. bovis*. Differences in polyketide synthesis and transport are also predicted due to differences in *pks* and *mmpSL* genes between *M. tuberculosis* and *M. bovis.* Differences in lipid profiles of these two variants provide further evidence of altered cell surfaces (Fig. 5).

In addition to pH and desiccation, *M. bovis* shows increased resistance to heat (Fig. 4B). The reason for this phenotypic difference from *M. tuberculosis* also could be due to differences in gene regulation. The transcriptional regulatory gene *whiB6* shows a

30.5 fold increased level of expression in *M. bovis* compared to *M. tuberculosis* in logarithmic growth (42). In *M. tuberculosis whiB6* has been shown to be up-regulated in response to heat-shock and other stress conditions (18). This suggests *whiB6* could be involved in a stress response and may have a role in the difference in susceptibility to heat that we observe here between *M. bovis* and *M. tuberculosis*. The elevated levels of *whib6* may predispose *M. bovis* to greater resistance to heat. Variations in cell envelope composition may also affect the resistance of mycobacteria to heat.

M. bovis has elevated levels of HupB compared to *M. tuberculosis* (Fig. 6). The HupB protein is alternatively known as both Hlp ("histone-like protein") and as LBP ("laminin-binding protein"). Recently it has been found that *hupB* is up-regulated in mycobacteria during iron limitation, cold shock, and anaerobiosis (29, 45, 53). Histone-like proteins in *Escherichia coli* play roles in viability during stationary phase (7), resistance to cold, heat, and UV light (30, 34, 48), DNA repair (6), and cell and colony morphologies (15, 38, 48). Therefore it is possible that elevated levels of HupB in *M. bovis* contribute to it's enhance stress resistance compared to *M. tuberculosis*.

HupB is not the only protein up-regulated in *M. bovis.* One protein band with more intense staining in *M. bovis* lysates contains a mixture of conserved hypothetical proteins Mb0222 (Rv0216) and Mb3102c (Rv3075c). Rehren *et al.* have reported that microarray analysis shows Rv0216 is up-regulated 11 fold in *M. bovis* compared to *M. tuberculosis* during the logarithmic phase (42). Mb2900 (MPB70; Rv2875) is another protein more prevalent in *M. bovis*. This protein is a known secreted, immunogenic mycobacterial protein (21, 32). MPB70 is reported to be synthesized at higher levels in *M. bovis* relative to all other members of the *M. tuberculosis* complex (22, 31, 42). This

elevated level of MPB70 has recently been shown to result from a mutation in an anti-sigma factor gene in *M. bovis* (43), adding further evidence that many phenotypic differences between *M. bovis* and *M. tuberculosis* may be due to differences in gene regulation rather than differences in structural genes (17).

In general, *M. bovis* was more resistant to stress of short duration (desiccation, heat, and low pH) compared to *M. tuberculosis*. Due to extensive genomic similarity, such differences could be attributed to differential gene regulation. Work is underway to examine the role *hupB* in the stress responses shown here.

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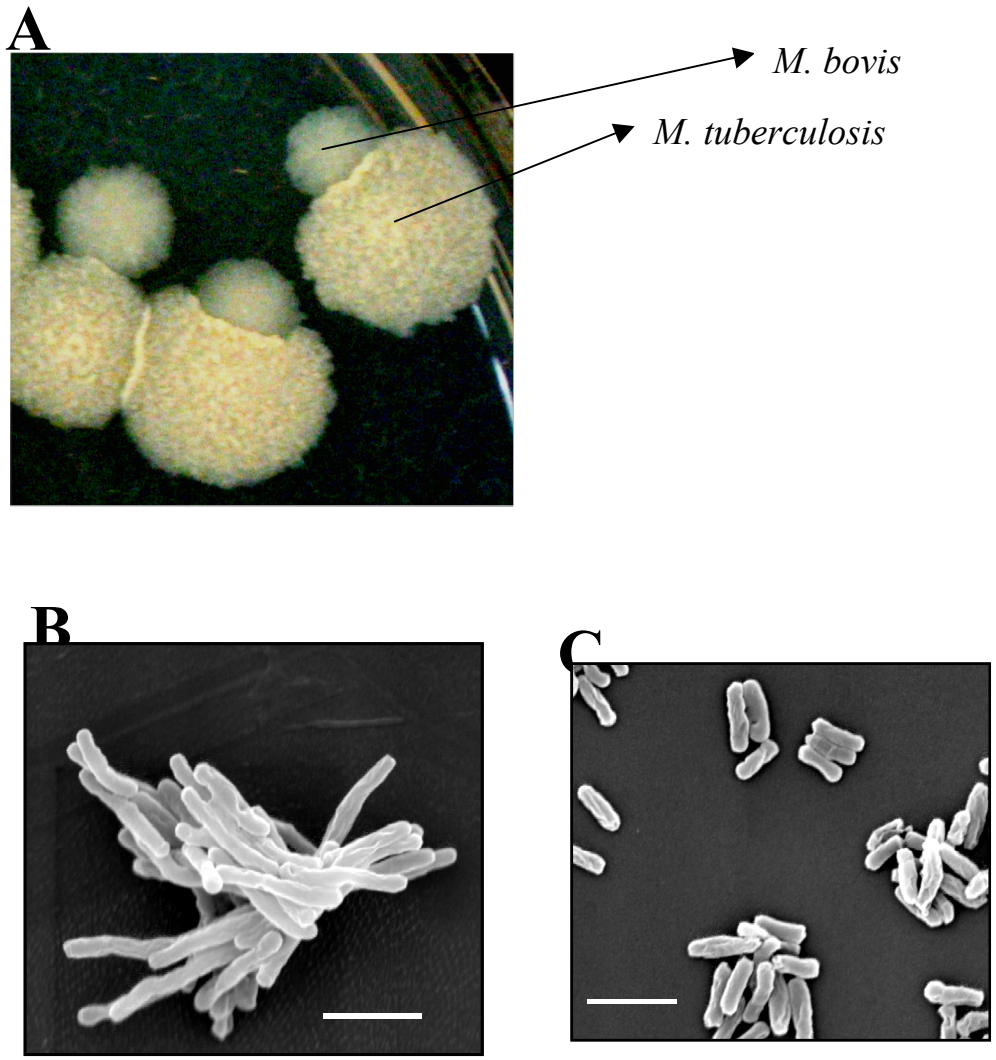

Figure 1. Morphologies of mycobacterial colonies and cells. (A) Five-week-old colonies for *M. tuberculosis* strain H37Rv and *M. bovis* strain 95-1315 are shown. *M. tuberculosis* colonies are markedly rougher and larger than *M. bovis* colonies. (B) SEM of *M. tuberculosis* and (C) *M. bovis* cells. Bars = 2 μm

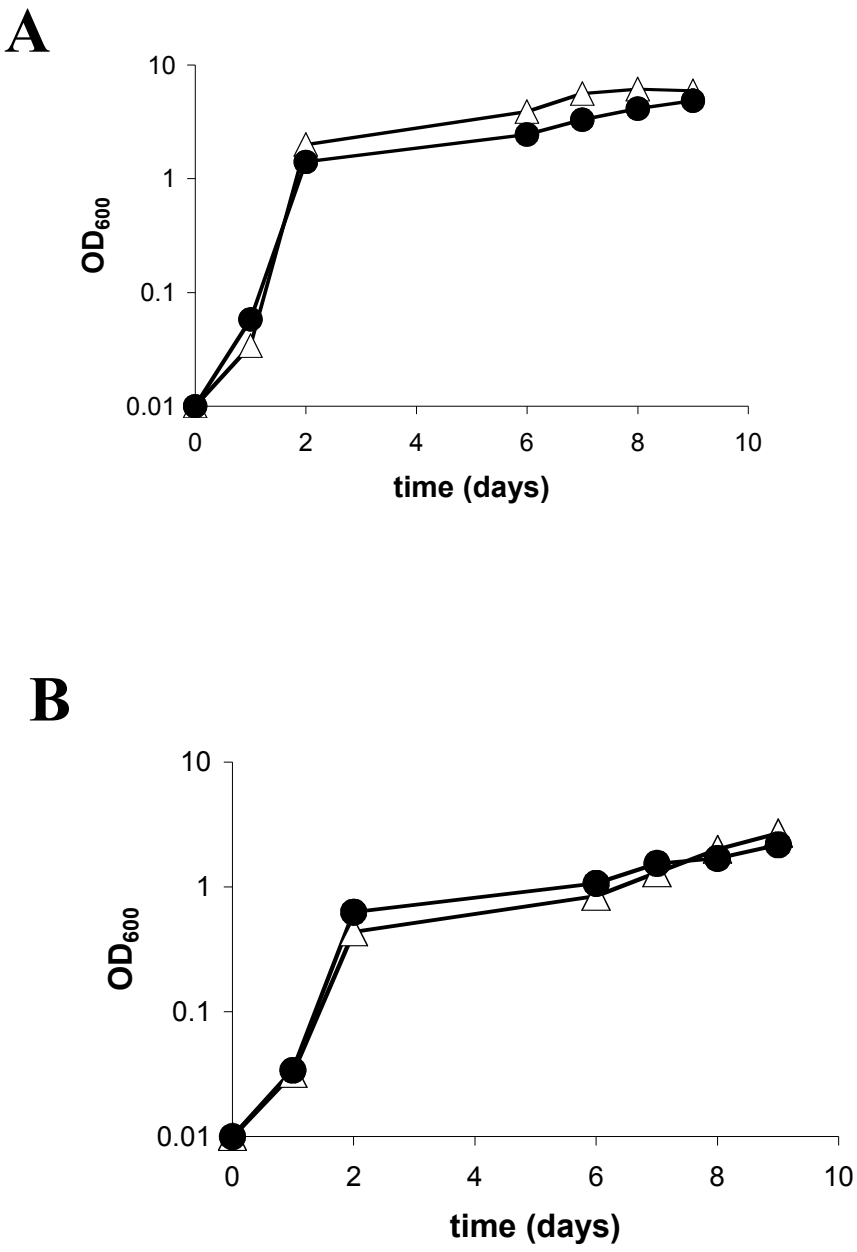

Figure 2. Growth dynamics of *M. tuberculosis* and *M. bovis*. Absorbance (OD_{600}) values were measured for *M. tuberculosis* (Δ) and *M. bovis* (●) cultures grown in 7H9+ADC+Tween+glycerol (A) or Sauton's medium+Tween (B).

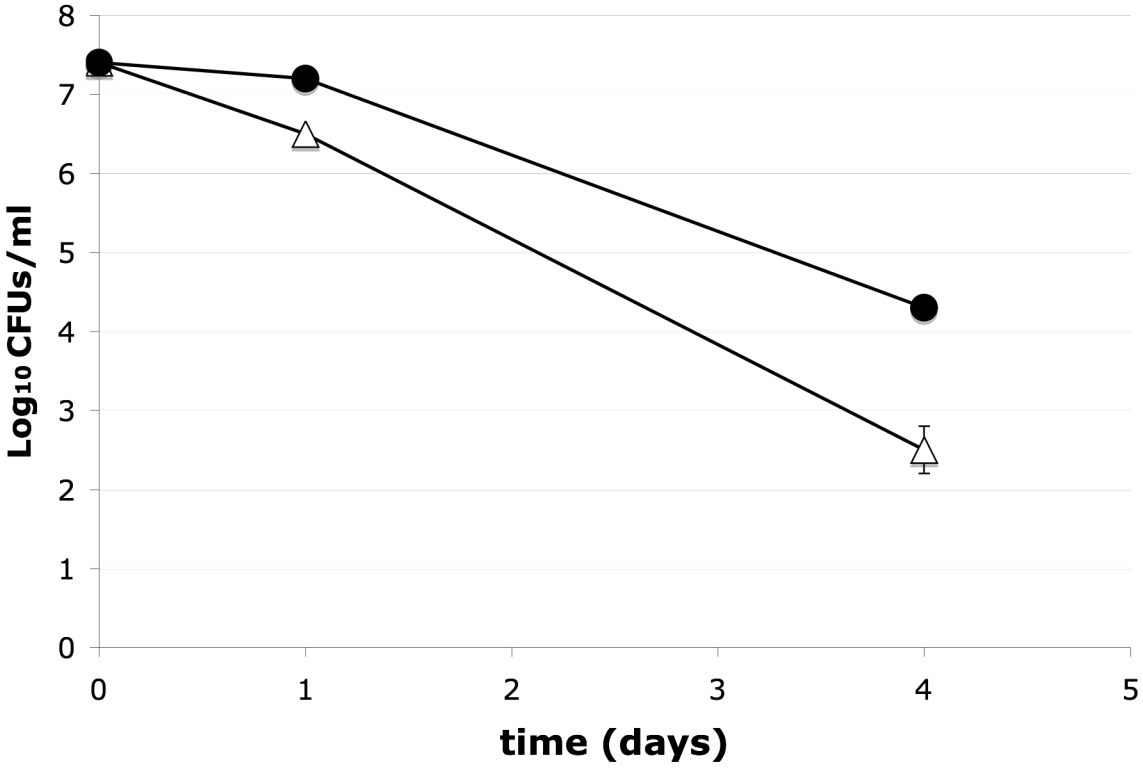

Figure 3. Analysis of mycobacteria for survival at pH 3.5. cfus ml^{-1} from *M. tuberculosis* (Δ) and *M. bovis* (●) were assayed for tolerance to pH 3.5 over a 4-day period.

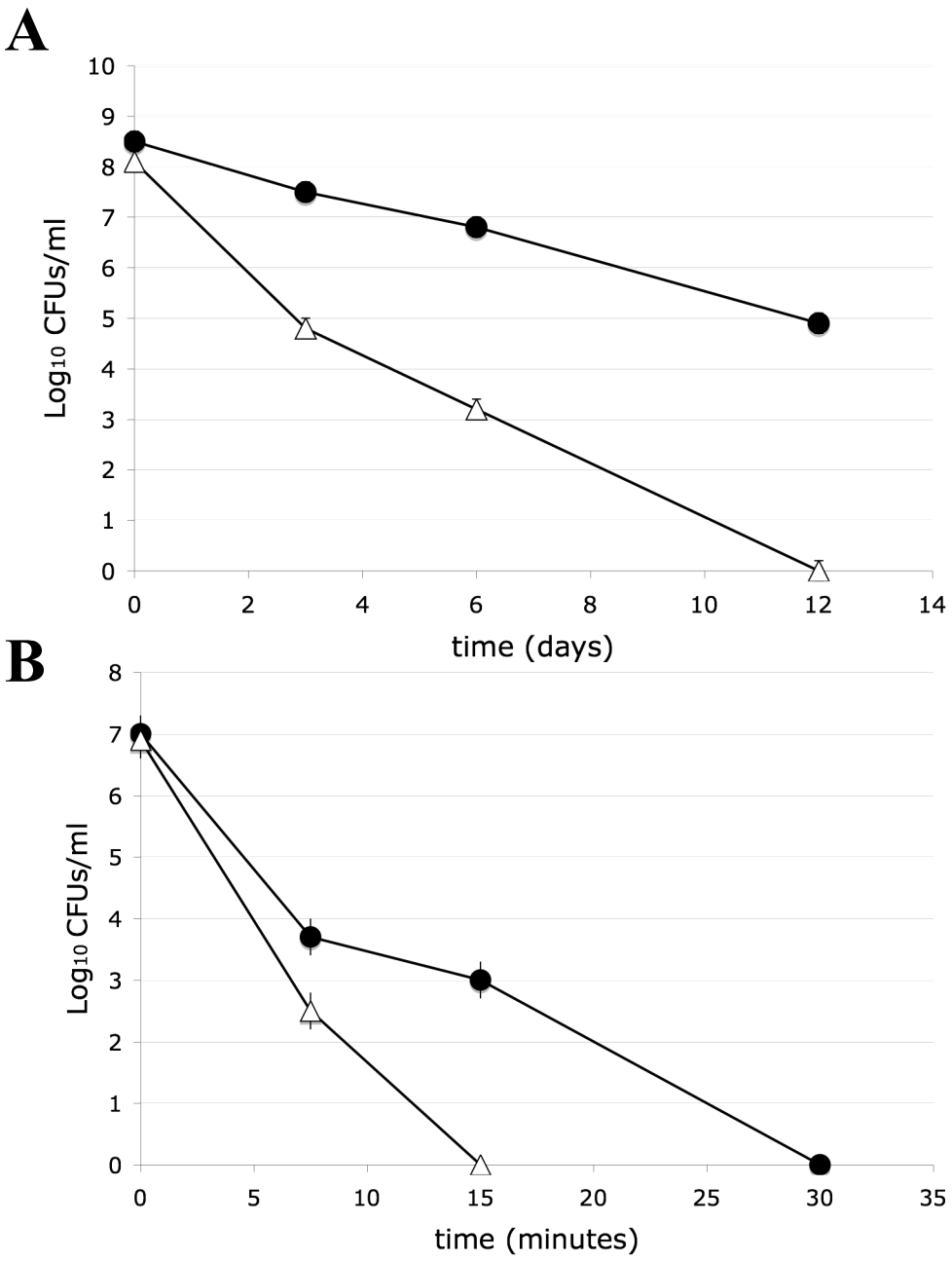

Figure 4. Viabilities of mycobacteria subjected to desiccation and heat. cfus ml^{-1} from *M. tuberculosis* (Δ) and *M. bovis* (●) are shown. (A) Cells were dried on cover slips and resuspended in 7H9 after various lengths of incubation in the dark at room temperature. (B) Cells were compared for survival after exposure to 53°C for increasing lengths of time.

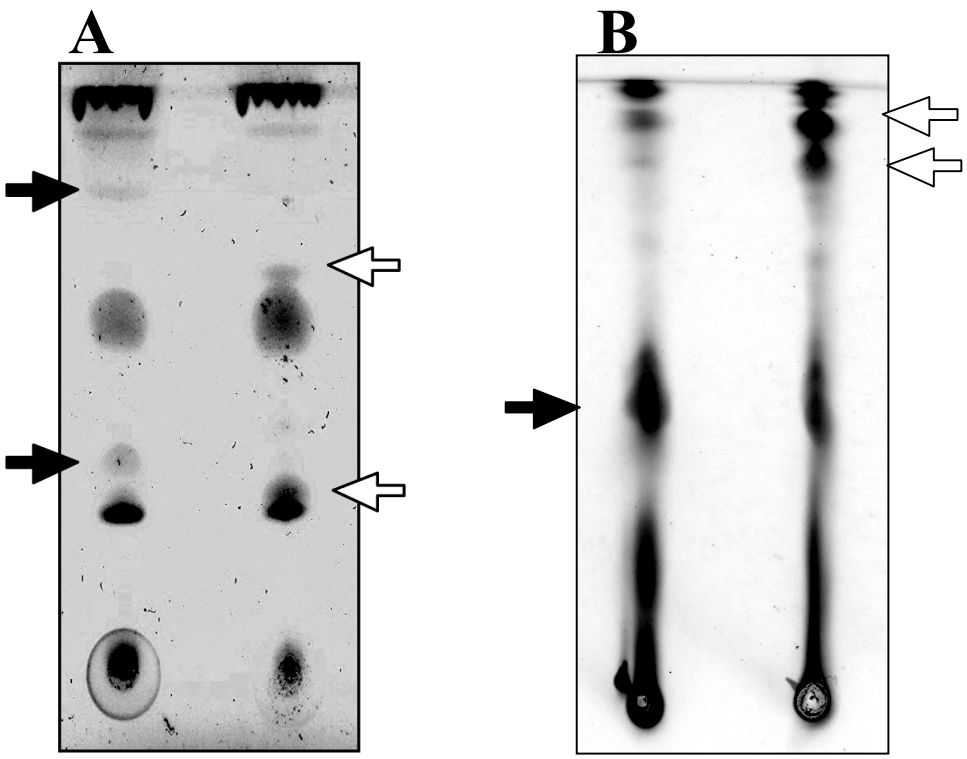

Figure 5. Analysis of cell envelopes of mycobacteria grown in 7H9-ADC-Tween. (A) Polar lipids and (B) apolar lipids for *M. tuberculosis* (lanes 1), *M. bovis* (lanes 2) were compared. Closed arrows indicate molecules with higher levels in *M. tuberculosis*, and open arrows indicate molecular species more prevalent in strains *M. bovis*.

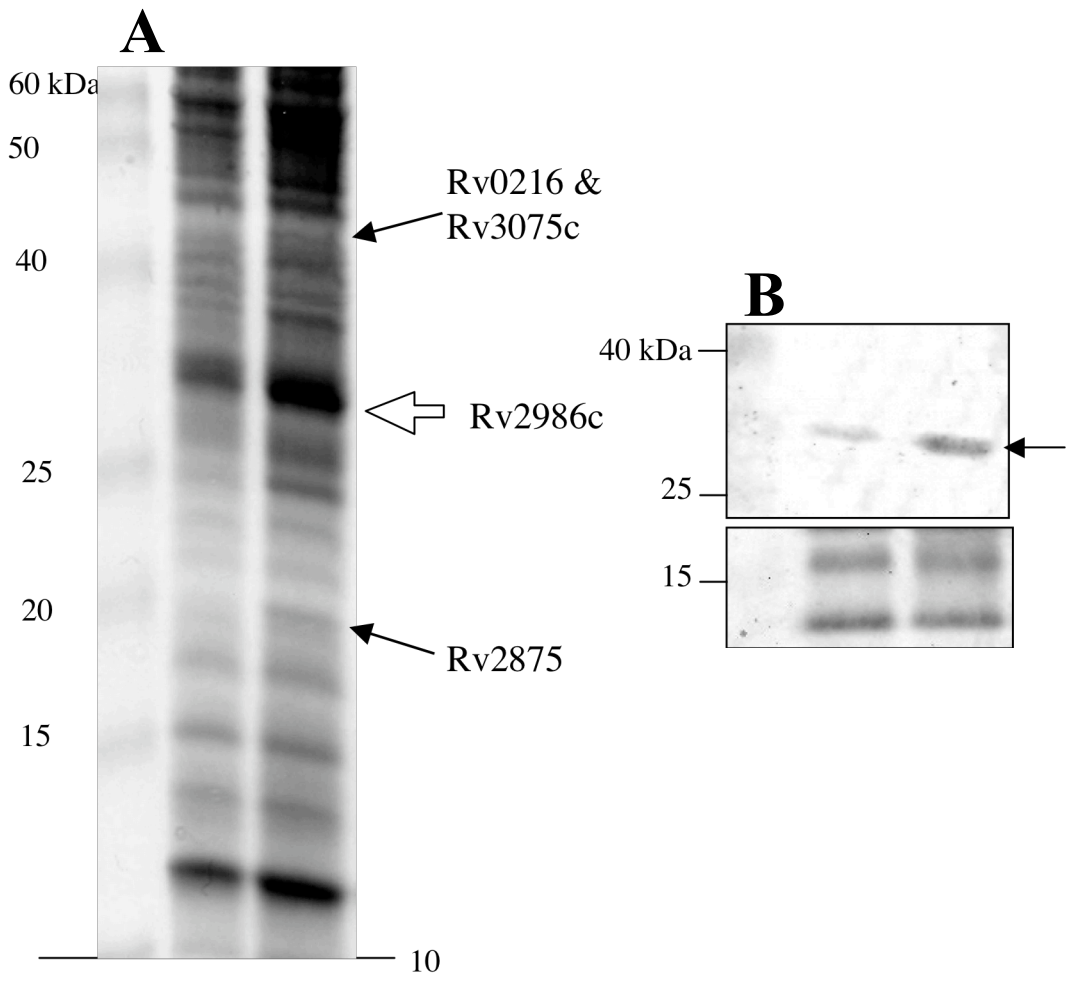

Figure 6. Protein comparisons of *M. bovis* and *M. tuberculosis*. (A) Coomassie-stained 12% SDS-PAGE gel of lysate from cells. Lanes: (1) molecular weight marker, (2) *M. tuberculosis* and (3) *M. bovis.* (B) Western blot analysis of HupB. Lysates were probed with antibodies to either HupB (upper half; arrow) or to whole-cell *M. tuberculosis* lysates (lower half). Lanes: (1) molecular weight marker, (2) *M. tuberculosis* and (3) *M. bovis.*

CHAPTER THREE

Deletion of the histone-like protein Hup results in increased sensitivity to UV exposure and isoniazid in *Mycobacterium smegmatis.*

Danelle C. Whiteford[1], Dalia Lau-Bonilla[1], Kriti Arora,[1]

and John L. Dahl[2]*

School of Molecular Biosciences, Washington State University, Pullman, Washington 99164[1], Department of Biology, University of Minnesota Duluth, Duluth, Minnesota 55512[2]

*Corresponding author. Mailing address: University of Minnesota Duluth, School of Biology, 207 SSB, 1035 Kirby Dr, Duluth, MN 55812. Phone: (218) 726-7264. E-mail: jldahl@d.umn.edu.

ABSTRACT

Protection and adaptation to environmental stress is an important survival characteristic of any bacterial species. As a soil dwelling saprophyte, *Mycobacterium smegmatis* is naturally exposed to factors such as ultraviolet light and rounds of freezing and thawing that occur in a temperate climate. Numerous studies in *Escherichia coli* have linked histone-like proteins with stress resistance and adaptation. We hypothesized that the histone-like protein Hup might likewise be involved in the stress response of *M. smegmatis*. Therefore, we examined the role of Hup on the ability of *M. smegmatis* to survive common environmental stresses. To accomplish this, the *hup* gene was inactivated and this mutant was compared with an isogenic strain containing a functional *hup*. It was found that the *M. smegmatis* Δ*hup* strain was more susceptible to UV exposure and the stress created by repeated freeze/thaw cycles. In addition, loss of Hup altered the colony morphology and allowed the organism to grow dispersed in the absence of a detergent, suggesting changes in the cell wall composition. As cell wall changes could affect the permeability to certain antibiotics, the susceptibility of *M. smegmatis* Δ*hup* to kanamycin, rifampicin, ethambutol and isoniazid was tested. *M. smegmatis* Δ*hup* was more susceptible to isoniazid, but loss of Hup did not affect susceptibility to the other antibiotics tested. This suggests the increased sensitivity of *M. smegmatis* Δ*hup* to isoniazid was unlikely the result of alterations in cell envelope permeability.

INTRODUCTION

Mycobacterium smegmatis is a nonpathogenic fast-growing mycobacterial species that is a popular model for studying slow-growing mycobacterial pathogens. It is a soil dwelling species and consequently is exposed to environmental stresses that without adaptive survival mechanisms would be lethal. The lipid-rich cell envelope of mycobacteria does provide some protection against common stressors by acting as a barrier to the environment (5). However, specific stress responses allow the cell to sense an environmental insult and alter gene regulation to produce cellular and physiological changes designed to increase resistance or to allow adaptation. In eubacterial species a class of "histone-like" proteins have been linked to survival of numerous environmental stresses such as ultraviolet light (16, 17), cold shock (32), heat shock (32), and lack of nutrients (7).

Bacterial histone-like proteins are small, highly-basic proteins that are typically found in high abundance in cells (31). They display similarity to eukaryotic histones in their ability to bind DNA and influence gene regulation (1, 21, 29). *M. smegmatis* possesses a histone-like protein called Hup (also known as Hlp and MDP1) (13, 14) that contains an N-terminal domain with sequence homology to the HU proteins of *E. coli* (18), one of the most well studied families of histone-like proteins in bacteria. Hup differs from other bacterial histone-like proteins in that it contains a C-terminal domain composed of prolines, alanines, and lysines, resembling the eukaryotic histone H1 family of proteins (18). Both the C-terminal and N-terminal domains of Hup are involved in binding DNA with high affinity (18). Homologs to the *M. smegmatis* Hup have been

found in most mycobacterial species, including the major pathogenic species, *M. tuberculosis* and *M. leprae* (26).

In *M. smegmatis* Hup was identified as a major protein species upregulated during bacterial dormancy (14). It was also identified as the major cold-shock-induced protein in *M. smegmatis* (26). Its homolog in *M. tuberculosis,* HupB was found to be upregulated by iron limitation (34) and by the stringent response, a survival mechanism triggered by nutrient deprivation (10). Although HupB in *M. tuberculosis* is predicted to be an essential gene (25), Lee *et al*. were able to generate a *hup* knockout in *M. smegmatis* (14). This deletion was found to have no effect on viability of two-week-old cultures (14), but it did affect the ability of *M. smegmatis* to adapt to cold shock (26).

The frequent induction of *hup* homologs in response to stress and the apparent importance of Hup in cold shock adaptation suggest Hup may have a role in stress resistance or adaptation. Due to its ability to bind DNA and repress transcription *in vitro,* it has been suggested that Hup could be involved in regulating gene expression during a stress response (18). In this study we examined the effect of a *hup* deletion upon the long-term survival of *M. smegmatis* in liquid culture and upon resistance to additional environmental and chemical stresses. We found that *M. smegmatis* Δ*hup* was more susceptible to UV exposure and to the front-line antibiotic isoniazid. Likewise, *M. smegmatis* Δ*hup* demonstrated a decrease in freeze tolerance. However, Hup did not appear to play a role in long-term survival of cells in starvation conditions.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions. All *M. smegmatis* liquid cultures were grown in 7H9 (Difco) medium supplemented with 0.05% Tween 80 and 0.2% glycerol, unless otherwise stated. For the long-term viability assay, cultures were gradually starved in 1.3-ml aliquots of 7H9+Tween 80 in Sarstedt tubes with gas-impermeable O-rings and maintained in a 37°C stationary incubator. At various time points, cells were gently vortexed with 0.1-mm diameter, sterile glass beads and serially diluted on 7H11 agar plates to determine colony forming units (CFUs) ml^{-1}.

Deletion of *hup* from *M. smegmatis* mc^{2}155. Deletion of *hup* from *M. smegmatis* mc^{2}155 was performed by allelic replacement. Briefly, an approximately 700-bp PCR product (forward primer HusmF1:agtcca**cttaag**cggcttccgggtcgtgatct, reverse primer HusmR1:agtcca**tctaga**cgatggtgtcgacgacgttctcc), directly upstream of the *M. smegmatis hup,* was cloned into the AflII and XbaI restriction sites on pYUB854 (3) on one side of a hygromycin (Hyg) resistance cassette. Another approximately 700-bp PCR product (forward primer HusmF2:agtcca**ccatgg**agaaggccgccgccaagaag, reverse primer HusmR2:agtcca**agatct**gaccggtcgacaccaagacggta) from directly downstream of *hup* was inserted into the SpeI and HindIII restriction sites on the other side of the Hyg cassette. The resulting plasmid was named pDW150. A marker cassette containing P_{Ag85}-*lacZ*P_{hsp60}-*sacB* was removed from pGoal17 (22) and inserted into the PacI site of pDW150. This plasmid, named pDW151, was then treated with 100 mJ/cm^2 UV light and electroporated into *M. smegmatis* mc^{2}155.

Following plating on 7H11 medium supplemented with 40 μg/ml X-gal and 50 μg/ml Hyg, transformants were selected that represented a single-crossover event (blue-Hyg^R colonies). The single-crossover transformants were then patched onto 7H11 plates with 50 μg/ml Hyg and 10% sucrose to screen for loss of sucrose sensitivity and the *lacZ* maker. The resulting Hyg^RSuc^I*lacZ*- colonies were considered double recombinants and verified for loss of *hup* by PCR and Southern Blot analyses.

Complementation of *hup* was performed using the integrative plasmid pMV306 (28). An 833-bp PCR product (forward primer HusmCF:cgg**ggtacc**ccgaattcgccgcccacct, reverse primer HusmCR:cgc**ggatcc**cggcgtgccttttgcggaat) was generated to include both the entire *hup* gene and approximately 150 bp upstream of the start site. This PCR product was then inserted into the KpnI and BamHI sites on pMV306 to create the construct for complementation called pDW160. This construct was electroporated into *M. smegmatis* Δ*hup* where it integrated onto the chromosome to generate *M. smegmatis* Δ*hup*::pMV306/*hup.*

Confirmation of *hup* deletion from *M. smegmatis*. To confirm the loss of *hup,* Southern blot analysis was performed as previously described (24). Genomic DNA from both *M. smegmatis* mc^2155 and Δ*hup* was digested with NotI and ScaI for 24 hr before separating by gel electrophoresis and transferring to a nylon membrane. A 306-bp product (forward primer HusmPrF:cgacatcttccgggggaacg, reverse primer HusmPrR:ccggatcaggccgggagagt) was amplified from the region directly upstream of the *hup* gene on the *M. smegmatis* chromosome. This product was then used as a template for a PCR reaction using a Digoxigenin (DIG) labeling kit (Roche). This reaction

incorporated DIG-labeled dUTP into the PCR product generating a probe for the Southern Blot. This probe was allowed to hybridize membrane bound DNA and detection of the probe was performed using a NBT/BCIP chromogenic reaction, as described by the manufacturer.

In addition to Southern blot analysis, deletion of *hup* from the *M. smegmatis* chromosome was confirmed by PCR using primers that amplified a 208-bp internal region of *hup* (forward primer HusmIntF:cgggttcggtgtcttcgagca, reverse primer HusmIntR:ggccttcttggcgggaccag). As *M. smegmatis* Δ*hup* was generated using an allelic replacement strategy, this PCR reaction was used to confirm the absence of the internal region of *hup* from the chromosome. Coomassie stained, SDS-PAGE of late-logarithmic grown cultures was used to confirm the loss of the Hup protein from *M. smegmatis* Δ*hup*.

UV exposure. One-ml aliquots of *M. smegmatis* cells grown to mid log (OD_{600} 0.6) were placed in 10-mm sterile Petri dishes and exposed to increasing amounts of UV (5.0 to 15.0 J/m^2) using a Biorad GeneLinker UV box. Serial dilutions of treated cells were immediately performed and plated onto 7H11. Results were reported as percent survival compared to samples untreated with UV light.

Isoniazid Sensitivity Assay. Sensitivity to isoniazid (INH) was examined using both the Kirby-Bauer technique and the determination of the minimum inhibitory concentration (MIC). First, discs soaked with concentrations of INH from 0-20.0 μg/ml were placed on a lawn of cells swabbed from early log cultures (OD_{600} 0.2). After 48 hr of incubation, the zones of inhibition were measured in centimeters. The MIC was

determined as previously described (33). Briefly, cultures were grown to OD_{600} 0.2. These samples were then diluted 1000 fold and 50-μl aliquots were placed in triplicate into each column of a 96-well round bottom plate. Decreasing concentrations of INH (80-0.156 μg/ml) were then added to the wells in 50-μl aliquots. Following a 48-hr incubation step, wells were examined using a dissecting scope for growth or no growth. Sensitivity to rifampicin, ethambutol, and kanamycin was also tested using the two methods described above.

Freeze-thaw assay. *M. smegmatis* cultures were grown to OD_{600} 0.6 in 7H9+Tween 80. 200-μl aliquots were transferred to 1.5-ml eppendorf tubes and frozen at -80°C before removing and immediately thawing in a 37°C water bath. This pattern was repeated 20 times before cells were serially diluted and plated on 7H11. CFUs were enumerated after 4-days growth and the percent survival calculated using the results obtained with untreated cells.

RESULTS

Deletion of *hup* from *M. smegmatis* mc^{2}155. Deletion of the *hup* gene was completed by the replacement of *hup* with a Hygromycin resistant (Hyg^R) cassette. This replacement was confirmed by Southern blot analysis (Fig. 1A). The DIG-labeled probe bound to a 2.125-kb band in the DNA from *M. smegmatis* mc^{2}155 and a 3.537-kb band in the mutant DNA (Fig. 1A*)*. The larger product in the mutant strain corresponds to the replacement of the *hup* gene with the Hyg^R marker.

PCR analysis showed the absence of an internal region of *hup* in the *M. smegmatis* Δ*hup* chromosome (Fig. 1B). A non-specific PCR product (upper band) served as an internal control. Loss of the Hup protein was also demonstrated by SDS PAGE (Fig. 1C). Late logarithmic cultures of *M. smegmatis* Δ*hup* were missing an approximately 30-kDa protein. Despite its apparent size of 21 kDa, Hup has been shown to migrate slower on SDS-PAGE due to its highly basic nature (14, 26). Complementation of *hup* in strain *M. smegmatis* Δ*hup* using the integrative plasmid pDW160 results in the reappearance of the 30-kDa protein.

***M. smegmatis* Δ*hup* demonstrates altered colony morphology.** *M. smegmatis* mc^{2}155 forms a rough colony, with ridges forming across the entire surface (Fig. 2A). However, colonies of *M. smegmatis* lacking Hup have a smoother morphology (Fig. 2B). While ridges do form on the edges, the center of the *M. smegmatis* Δ*hup* colony remains flatter and lacks the extensive ridges seen in the wild-type strain. The complemented

mutant has the typical rough appearance of mc^{2}155 (data not shown). Smooth colony phenotypes in *M. smegmatis* have been previously reported and linked with altered cell wall hydrophobicity (2, 6). It is widely known that *M. smegmatis* mc^{2}155 requires Tween 80 to grow dispersed in liquid culture. Without Tween 80 the hydrophobic nature of the *M. smegmatis* mc^{2}155 cell wall causes the cultures to aggregate in liquid. When *M. smegmatis* Δ*hup* was grown in the absence of the detergent, the cells were able to grow dispersed in solution without the clumping that was seen in *M. smegmatis* mc^{2}155 (Fig. 2C).

Growth and long-term survival of *M. smegmatis* Δ*hup.* Growth of *M. smegmatis* Δ*hup* in 7H9+Tween 80 was similar to that of *M. smegmatis* mc^{2}155 when grown at 37°C (Fig. 3A) or 45°C (data not shown). Lee *et al.* examined the ability of a *M. smegmatis hup* mutant strain to survive two weeks of bacterial dormancy by sustaining cultures in a low oxygen environment (14). As mycobacteria are known to survive years of starvation (9, 19), we decided to examine long-term viability for up to 8 months. Loss of *hup* had no effect on long-term viability in these 8-month oxygen starved cultures (Fig. 3B).

***M. smegmatis* Δ*hup* has increased sensitivity to UV exposure.** A previous study showed that lack of the histone-like protein HU from *E. coli* lead to an increase in the sensitivity to UV light (16). This was due in part to an involvement in DNA repair processes (17). As a DNA-binding protein, Hup could have an involvement in DNA repair, which might be demonstrated by alterations in UV sensitivity upon deletion of the

hup gene. Deletion of *hup* from *M. smegmatis* did result in an increase in sensitivity to UV light (Fig. 4). When exposed to up to 15 J/m^2 UV, *M. smegmatis* Δ*hup* had an 8-fold decrease in survival compared to *M. smegmatis* mc^{2}155 or the complemented strain Δ*hup*::pMV306/*hup*.

***M. smegmatis* Δ*hup* has increased sensitivity to isoniazid.** Previous studies have linked alteration in cell wall composition and loss of a histone-like protein to alterations in antibiotic sensitivity (2, 8, 12, 15). In addition Lewin *et al*. saw an increase in the sensitivity to ampicillin when the concentration of Hup was decreased in *M. bovis* BCG (15). To determine whether loss of Hup generates a similar effect on antibiotic sensitivity in *M. smegmatis*, strains mc^{2}155, Δ*hup* and Δ*hup*::pMV306/*hup* were compared for sensitivity to various antibiotics. No differences were observed between the two strains for sensitivity to kanamycin, ethambutol, or rifampicin (data not shown). However *M. smegmatis* Δ*hup* showed increased sensitivity to varying concentrations of isoniazid (Fig. 5). The zones of isoniazid inhibition of *M. smegmatis* Δ*hup* were significantly larger than that of the wild-type or complemented strains, and the MIC for *M. smegmatis* Δ*hup* was 4-fold lower than for isogenic strains with a functional *hup* gene (Fig. 5).

***M. smegmatis* Δ*hup* has increased sensitivity to freezing.** A previous study linked *hup* with the ability of *M. smegmatis* to adapt to a cold shock of 10°C and resume growth (26). While this study looked at the ability of a *M. smegmatis hup* mutant to gradually acclimatize to a temperature of 10°C, here we tested the ability of *M.*

smegmatis Δ*hup* to survive a rapid temperature adjustment through repeated cycles of freezing and thawing (Fig. 6). After 20 rounds of freeze/thaw, *M. smegmatis* Δ*hup* had a 10-fold decrease in percent survival compared to mc^2155 and Δ*hup*::pMV306/*hup*.

DISCUSSION

In this report we have examined the role *hup* plays in stress tolerance and antibiotic sensitivity of *M. smegmatis*. We found that loss of Hup adversely affects the ability of the organism to survive UV radiation and freeze treatments. Additionally, *M. smegmatis* Δ*hup* was more susceptible to the antibiotic isoniazid.

Hup is a small, basic, DNA-binding protein that was first characterized in *M. smegmatis* as a major protein induced in response to cellular dormancy (14). Originally called HLP for "histone-like protein," Hup was found to share similarity to other prokaryotic histone-like proteins through its N-terminal domain and to the eukaryotic histone H1 proteins through its C-terminal domain (23). As a histone-like protein, Hup may play roles in DNA compaction and gene regulation necessary for stress survival. The ability of Hup to act as a transcriptional repressor in *M. smegmatis in vitro* hints at possible functions in regulating gene activity (18), although at present the full significance of Hup in *M. smegmatis* has not been elucidated. Hup homologs are found localized both to the cytosol and nucleoid (18, 23) and to the cell wall (27, 34). This dual localization suggests a complex and multi-functional role for these structurally unique mycobacterial histone-like proteins.

We have recently reported that loss of the DNA-binding protein Lsr2 from *M. smegmatis* produces a smooth colony morphology (2, 6, 8). The deletion of *hup* from the *M. smegmatis* chromosome also results in a smoother colony morphology (Fig. 2B). This suggests changes in the cell envelope composition. Using SEM, Katsube *et al*. showed that stationary phase cells of a *M. smegmatis hup* mutant had crenellated surfaces, unlike

the smooth cell surface seen in wild-type *M. smegmatis* (13). They also demonstrated that Hup was involved in the down-regulation of cell surface components in stationary phase (13). These results all indicate that loss of Hup affects the composition of the cell envelope. Such changes often coincide with differences in cell hydrophobicity (6, 20). A decrease in the hydrophobicity of *M. smegmatis* Δ*hup* is illustrated by its ability to grow dispersed in liquid media without the addition of the detergent Tween 80 (Fig. 2C). Given that Hup homologs are found localized to the cell envelope (13, 27) and that Hup can bind externally to *M. bovis* BCG (13), it is possible that Hup is involved in cell-to-cell aggregate in *M. smegmatis*. Lewin *et al.* found that after knocking down expression of the *hup* homolog in *M. bovis* BCG, there was a decrease in aggregation formation. (15).

As *hup* is up-regulated in response to bacterial dormancy, Lee *et al*. previously examined the effect of the loss of Hup on the long-term viability of oxygen-starved cultures left stationary for up to two weeks (14). While they did not observe any difference in viability between their *M. smegmatis hup* mutant and their wild-type strains, mycobacteria can exist in a dormant state for years (9, 19). Therefore we repeated this long-term starvation experiment for up to eight months. We confirmed that loss of *hup* did not affect long-term viability in oxygen-starved cultures (Fig. 3B). Both our results and those of Lee *et al*. (14) differ from those of Katusbe *et al*. who showed bacterial density of a *hup* mutant was slightly less than that of the wild-type in stationary phase (13). A recent study used an antisense approach to knock-down expression of the Hup homolog in *M. bovis* BCG (15). Once again it was found that in an atmosphere of low-oxygen, the strain expressing less *hup* did not demonstrate differences in growth

compared to the wild-type stain. However, differences in protein production were observed in oxygen-starved cultures suggesting Hup plays a role in protein expression of *M. bovis* BCG in hypoxic conditions. Further work is needed to determine whether the loss of Hup in *M. smegmatis* affects gene expression in conditions of low oxygen.

M. smegmatis Δ*hup* has an 8-fold decrease in survival when subjected to UV light (Fig. 4). In *E. coli*, deletion of the heterodimer formed by the histone-like proteins HUα and HUβ lead to an over 100-fold decrease in UV survival (16). This susceptibility to UV light in *E. coli* was attributed to deficiencies in RecA dependent recombinational DNA repair and in the induction of the SOS response (17). Although a comparatively smaller decrease was seen in the survival of the *hup* mutant (8-fold) in *M. smegmatis*, it is possible that the *M. smegmatis* Hup is also involved in DNA repair. A recent study demonstrated that Hup enhances DNA end-joining in *M. smegmatis in vitro* (18). While UV damage does not typically cause double strand breaks (DSB) in DNA, Hup could be involved in repairing any DSBs that occur during the repair of the UV caused pyrimidine dimers (4). An additional reason for the increased susceptibility to UV seen here in the Δ*hup* strain could be the alteration in the cell envelope structure. As a major barrier against the environment, changes in the cell envelope could affect UV sensitivity. Precedence for this exists in *Lactococcus lactis,* where the loss of genes involved in cell envelope composition leads to an increase in UV sensitivity (11).

M. smegmatis Δ*hup* was tested for its sensitivity to four antibiotics, isoniazid, rifampicin, ethambutol, and kanamycin. Compared to the wild-type strain, no differences were observed in the sensitivity to rifampicin, ethambutol or kanamycin. This result is similar to that seen in *M. bovis* BCG, where reducing the level of Hup did not reduce

sensitivity to rifampicin (15). However, the loss of hup from *M. smegmatis* did lead to a 4-fold increase in susceptibility to the antibiotic isoniazid (INH) (Fig. 5). INH is a front-line drug for the treatment of tuberculosis. It is a small hydrophilic prodrug that enters into the bacterial cell by passive diffusion where it is activated by the action of KatG (35). Activated INH can inhibit enzymes involved in the synthesis of mycolic acids (35). Further cellular damage can occur as a result of the action of reactive oxygen radicals, which are produced during prodrug activation (35). With the loss of Hup, *M. smegmatis* may be more susceptible to the free radicals produced by INH activation, suggesting a possible involvement between Hup and the resistance of *M. smegmatis* to oxidative damage. Alternatively, it is possible that alterations in the cell wall of *M. smegmatis* Δ*hup* could increase the permeability to INH. However, this does not seem likely since no Hup-dependent differences were seen in the sensitivities to the other antibiotics tested including ethambutol, a similarly small hydrophilic antibiotic.

Tolerance to freeze/thaw activity is a complex mechanism involving the accumulation of trehalose, the production of molecular chaperones, and the composition of the bacterial cell wall (30). *M. smegmatis* Δ*hup* has reduced tolerance to repeated freezing and thawing (Fig. 6). A previous study demonstrated the inability of a *M. smegmatis hup* mutant to resume growth after cultures were shifted from 37°C to 10°C (26). Our current study took the alternative approach of examining cellular viability after repeated rounds of freeze/thaw. As a major cold-shock-inducible protein, *hup* could be involved in stabilizing mRNA transcripts and allowing transcription initiation to occur after a cold shock (26). However, the method used here suggests a different role for Hup in freeze tolerance as the cells were rapidly shifted to a state of metabolic inactivity.

Therefore, the phenotype seen here could be due to post-translational differences in the cell envelope. In lactobacilli the fatty acid composition of the cell membrane has been linked to the tolerance of freeze/thaw (36), and it is possible the altered composition of the *M. smegmatis* Δ*hup* cell envelope lowers freeze tolerance.

The pleiotropic phenotype of the *hup* mutant suggests some regulator mechanism has been altered. It is possible that alterations in a single cell envelope moiety could be responsible for all of the phenotypes reported here but this seems less feasible than changes in the regulation of multiple genes due to loss of Hup. Such an involvement in wide scale gene regulation has been a point of frequent speculation (15, 18, 26). Further study of this possibility will be conducted through microarray comparisons of the wild-type and Δ*hup* strains.

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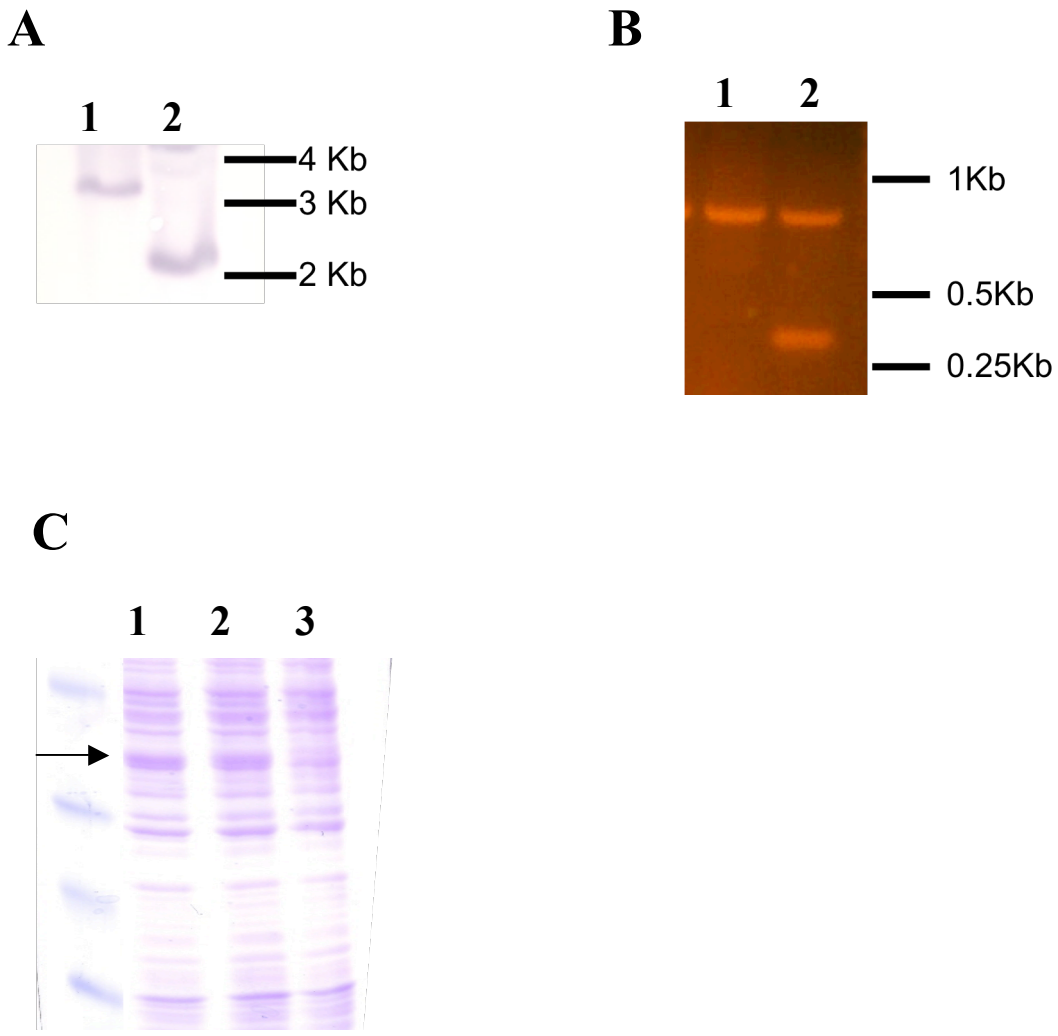


Figure 1. Confirmation of *hup* deletion in *M. smegmatis*. (A) Southern blot of Δ*hup* (lane 1) produces a product that is 1.4 kb larger than mc^{2}155 (lane 2). (B) PCR reveal loss of 200 bp internal region of *hup* gene from Δ*hup* (lane 1) that is present in mc^{2}155(lane 2). The upper band is a non-specific PCR product that serves as an internal control. (C) Coomassie stained 12% SDS-PAGE of logarithmic cultures demonstrates presence of Hup protein (arrow) in mc^{2}155 (lane 1) and Δ*hup*::pMV306/*hup* (lane 2) but absence in Δ*hup* (lane 3).

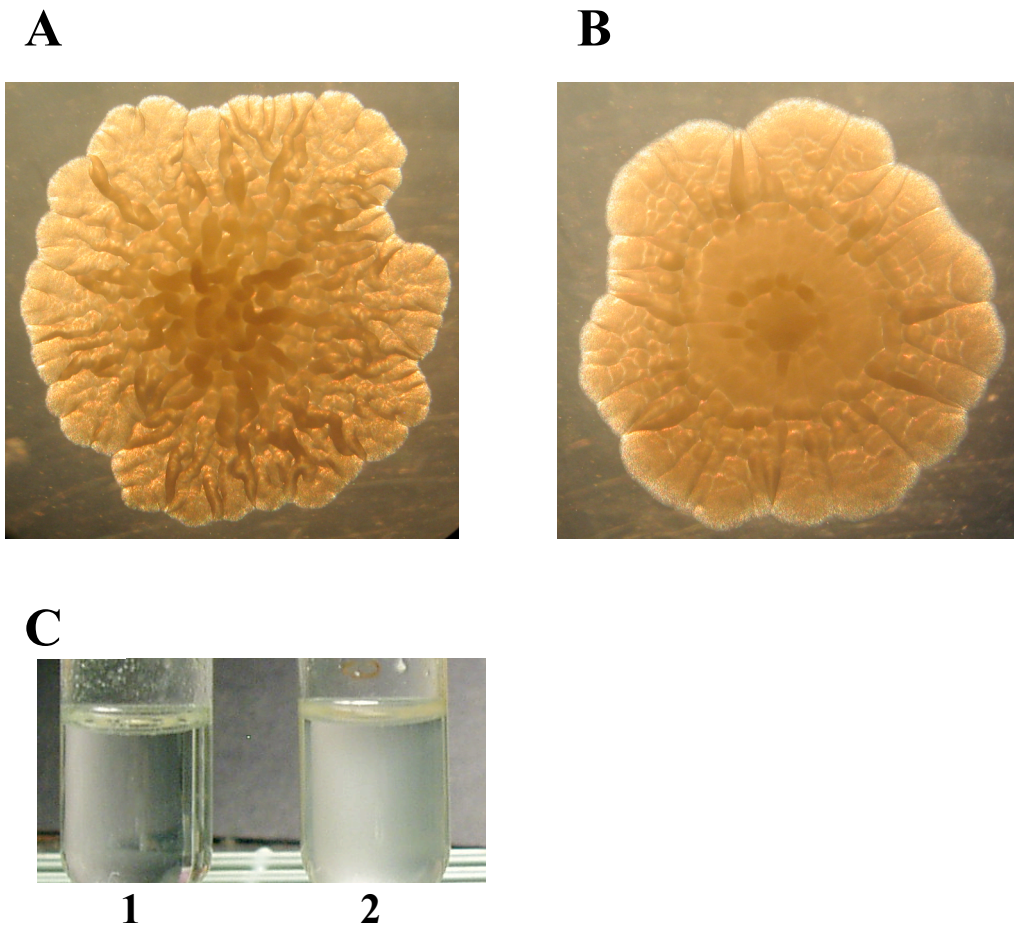


Figure 2. Morphology of *M. smegmatis* colonies and growth in absence of Tween 80. (A) mc^2155 and (B) Δ*hup* colonies demonstrate different morphologies grown on 7H11. (C) In the absence of Tween80, mc^2155 (1) is unable to grow dispersed in solution, while Δ*hup* (2) remains suspended in solution.

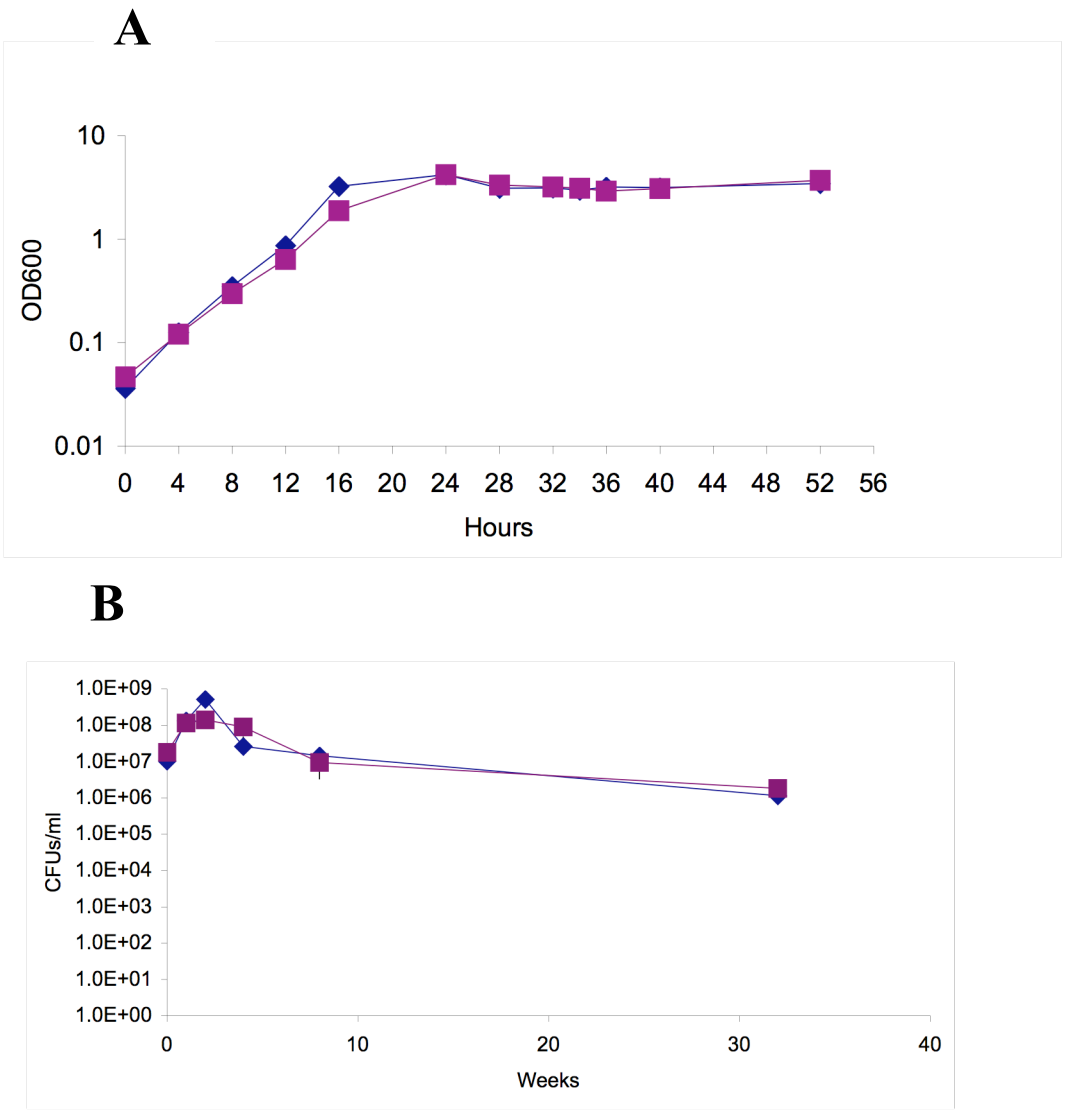


Figure 3. Growth curve and long-term survival of *M. smegmatis* Δ*hup*. (A) mc^{2}155 (diamonds) and Δ*hup* (squares) have similar growth rates in liquid 7H9. (B) After eight months stationary in 7H9 with little head space, mc^{2}155 (diamonds) and Δ*hup* cultures show no difference in viability.

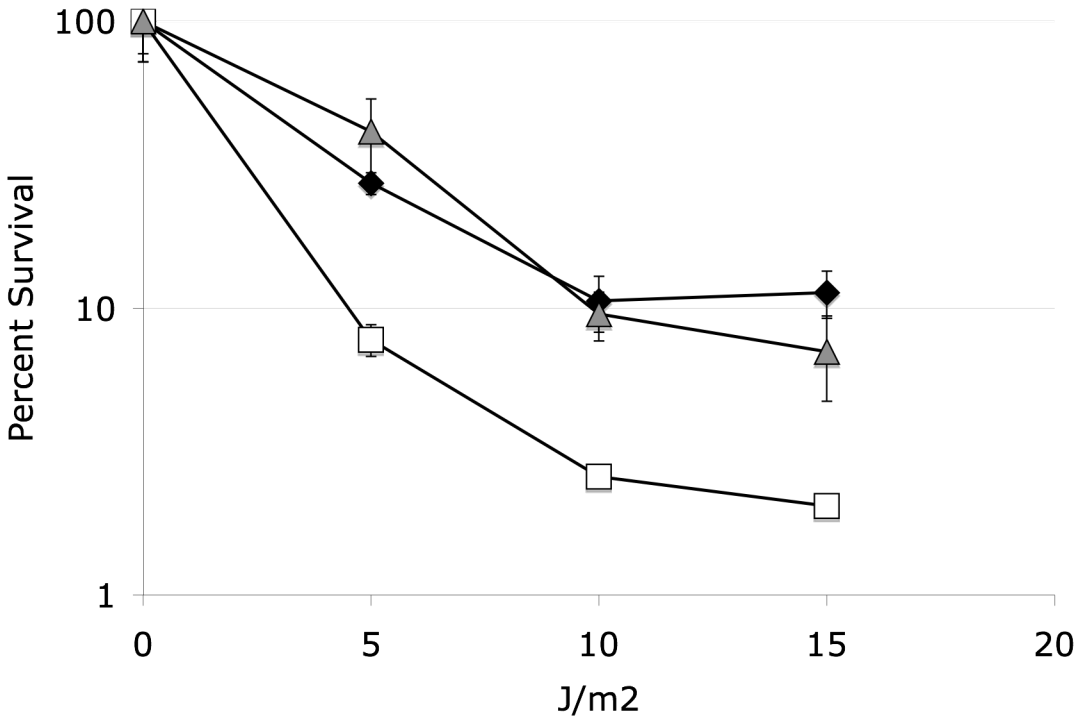
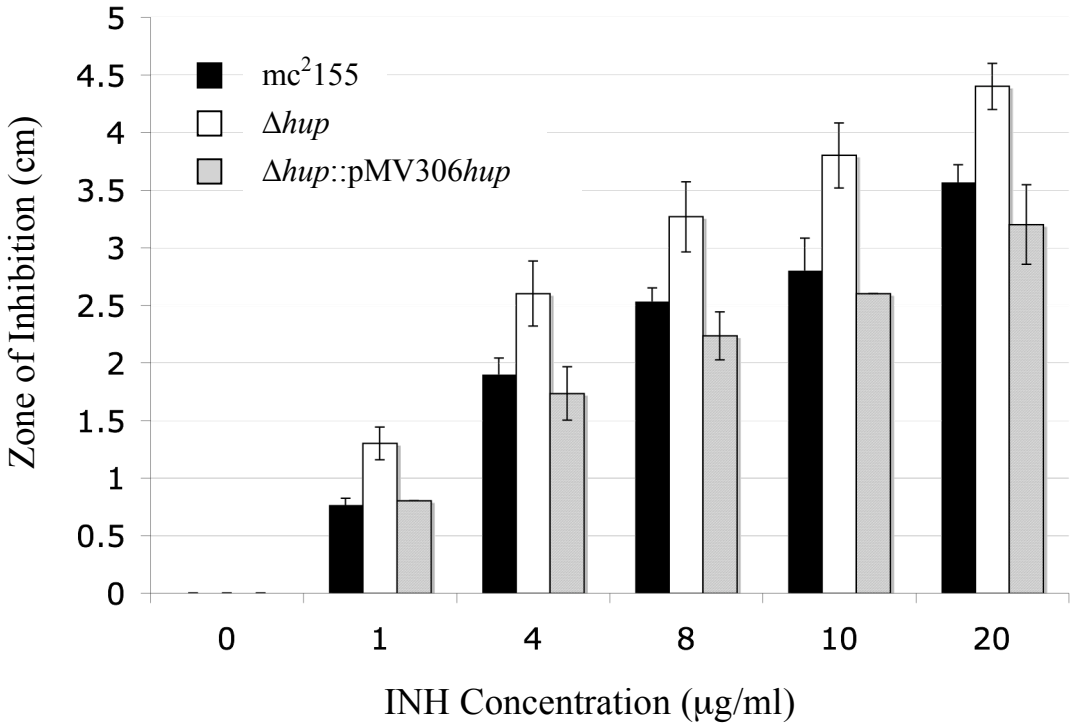


Figure 4. Survival of UV light exposure. *M. smegmatis* Δ*hup* (squares) has decreased survival compared to mc^{2}155 (diamonds) or Δ*hup::*pMV306/*hup* (triangles).

A



B

Strain	MIC
mc²155	10 μg/ml
Δ*hup::pMV306/hup*	10 μg/ml
Δ*hup*	2.5 μg/ml

Figure 5. Loss of *hup* confers INH susceptibility. (A) Zones of inhibition were measured around disks soaked with increasing concentrations of INH. Error bars represent standard deviations for three experiments. (B) The MIC determined for Δ*hup* was 4 times lower than that of the wild-type and complemented strains.

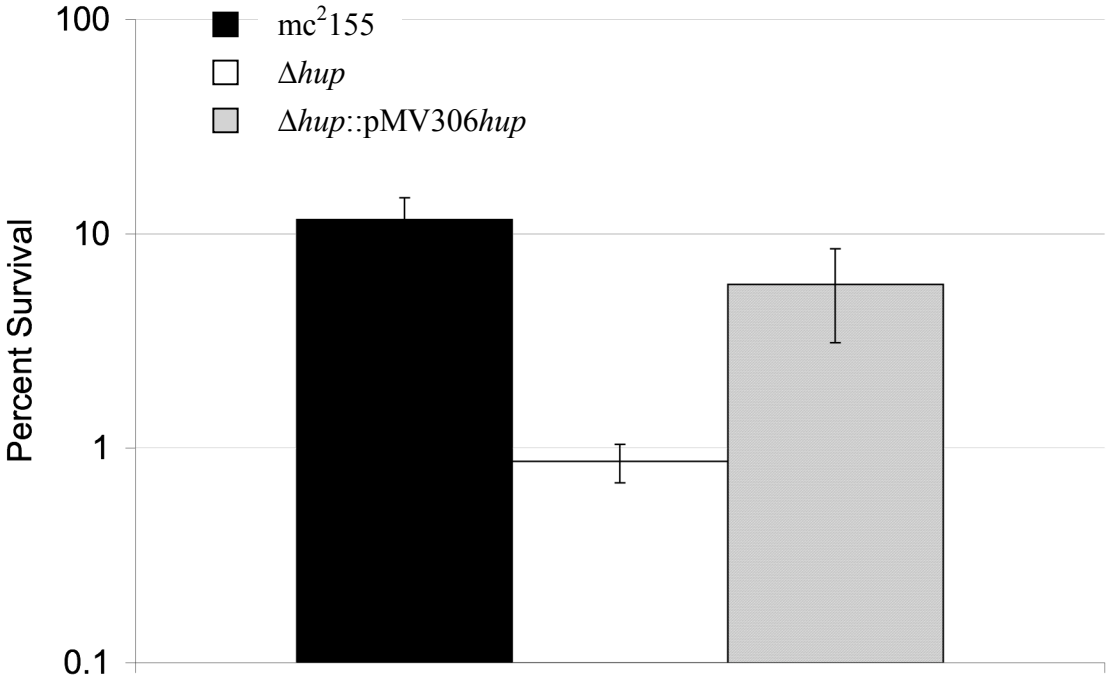


Figure 6. Viability after freeze/thaw treatment. *M. smegmatis* Δ*hup* has decreased viability compared to mc^{2}155 and Δ*hup*::pMV306/*hup* after repeated freeze/thaw treatments.

CHAPTER FOUR

Inactivation of *lsr2* results in a hyper-motile phenotype in *Mycobacterium smegmatis.*

Kriti Arora[1], Danelle C. Whiteford[1], Dalia Lau-Bonilla[1], Christine M. Davitt[2], and John L. Dahl[1]*

School of Molecular Biosciences, Washington State University, Pullman, Washington 99164[1] and Franceschi Microscopy and Imaging Center, College of Sciences, Washington State University, Pullman, Washington 99164[2]

*Corresponding author. Mailing address: Washington State University, School of Molecular Biosciences, Abelson Hall, Room 301, Pullman, WA 99164. Phone: (509) 335-7719. Fax: (509) 335-1907. E-mail: johndahl@wsu.edu.

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As second author, my contribution to this publication was to generate the DL2008 strain and the DL2008/pLsr2 strain. In addition, I performed the experiments comparing these strains to wild-type *M. smegmatis.*

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 strain of *Mycobacterium smegmatis*, called DL1215, which demonstrates marked deviations from the above-mentioned phenotypes. DL1215 arose spontaneously from a strain deficient for the stringent response (*M. smegmatis* Δrel_{Msm}) and is not a reversion to a wild-type phenotype. The nature of the spontaneous mutation was a single base-pair deletion in the *lsr2* gene leading to the formation of a truncated protein product. The DL1215 strain was complicated by having both inactivated rel_{Msm} and *lsr2* genes, and so a single *lsr2* mutant was created to analyze the gene's function. The *lsr2* gene was inactivated in the wild-type *M. smegmatis* mc^2155 strain by allelic replacement to create strain DL2008. Strain DL2008 shows characteristics unique from both the wild-type and the Δrel_{Msm} strains, some of which include a greatly enhanced ability to slide over agar surfaces (referred to here as "hyper-motility"), greater resistance to phage infection and to the antibiotic kanamycin, and an inability to form biofilms. Complementation of the DL2008 mutant with a plasmid containing *lsr2* (pLSR2) reverts the strain to the mc^2155 phenotype. Although these phenotypic differences allude to changes in cell surface lipids, no difference is observed in glycopeptidolipids, polar lipids, apolar lipids, or mycolic acids of the cell wall.

INTRODUCTION

Mycobacterium smegmatis is a fast-growing, saprophytic mycobacterial species. Although considered non-pathogenic, *M. smegmatis* provides a popular model for studying virulence mechanisms of slow-growing, pathogenic relatives such as *Mycobacterium tuberculosis* (9, 16, 37, 41) and *Mycobacterium leprae* (35, 42). An important aspect of mycobacterial pathogenesis is the ability of the pathogen to establish latent infections in hosts lasting for several years. Persistent *M. tuberculosis* bacilli in the host manifest drastic changes in gene expression that set the cells apart from actively growing tubercle bacilli (36, 40). One bacterial regulatory network that coordinates nutrient deprivation with adaptive metabolism is the stringent response. In mycobacteria this global regulatory system is controlled by a single gene called *rel*, and deletion of this gene in *M. tuberculosis* results in a severe defect in both long-term *in vitro* and *in vivo* survival (10, 30). We have recently reported that the *rel* gene of *M. smegmatis* (rel_{Msm}) is involved in the regulation of cellular and colony morphologies (9). As seen with *M. tuberculosis*, the stringent response is required for long-term survival of *M. smegmatis* in culture since the rel_{Msm} mutant readily dies over a month-long period while in stationary phase.

Here we report the appearance of a mutant strain, called DL1215, that arose spontaneously from the parental *M. smegmatis* Δrel_{Msm} strain. We selected for DL1215 by subjecting *M. smegmatis* Δrel_{Msm} cells to prolonged nutrient stress. This mutant does not represent a reversion to a wild-type phenotype, which is possible in bacteria deficient for the stringent response if suppressor mutations arise in their RNA polymerases (12).

DL1215 also does not represent a contaminant as its identity as *M. smegmatis* was confirmed by 16s rRNA sequencing.

The most striking phenotype of DL1215 is its ability to spread over soft-agar surfaces much faster (a trait referred to here as "hyper-motility") than either the wild-type *M. smegmatis* mc^2155 strain or the parental *M. smegmatis* Δrel_{Msm} strain. To our knowledge, this is the first report of a mycobacterial species demonstrating such a high rate of surface spreading motility. The genus *Mycobacterium* had been generally considered non-motile until Roberto Kolter's laboratory demonstrated the abilities of *M. smegmatis* and *Mycobacterium avium* to spread on solid surfaces (22). This ability of *M. smegmatis* to spread was shown to directly correlate with the presence of glycopeptidolipids (GPLs) in the cell wall. Strains deficient in biosynthesis, transport, or acetylation of GPLs were unable to spread, and they produced colonies with a rougher phenotype than the wild-type strain (22, 31, 32). However, we show here that this hyper-motility is independent of the GPL content of *M. smegmatis* and likely involves other cellular systems. This hyper-motility directly correlates with inactivation of the *lsr2* gene.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. A list of the bacterial strains and plasmids used in this study is shown in Table 1. Liquid cultures were grown in 7H9 (Difco) medium supplemented with 0.2% glycerol and 0.05% Tween 80, unless stated otherwise. *M. smegmatis* strains were transformed with plasmid DNA by electroporation, as previously described (41). Transformants were selected for on Middlebrook 7H11 (Difco) agar medium containing hygromycin (50 μg/ml) or kanamycin (25 μg/ml) where appropriate.

Generation of strain DL2008. Plasmid pKA0505 was constructed by first PCR amplifying the mutant *lsr2* gene from strain DL1215 using forward primer LSR2F (5'-GATCTGAGCGTTGTTGATAG-3') and reverse primer LSR2R (5'-GTACCTGCCGTCCACTCTAA-3') (10). The 652-bp PCR product contained 199 bp upstream of the start codon and 108 bp downstream of the stop codon and was cloned into the *EcoRI* site of pDrive. The mutant *lsr2* allele was then excised with *BamHI* and *NotI* and cloned into the *BamHI-NotI* site of the MCS in p2NIL to create pKA0504. The Hyg^R-P_{Ag85}-*lacZ*-P_{hsp60}-*sacB* marker cassette from pGOAL19 was released by *PacI* digest and cloned into the *PacI* site of pKA0504 to create pKA0505. *M. smegmatis* mc^2155 was transformed with pKA0505 and single cross-over events were selected as blue colonies on 7H11 agar plates with 50 μg/ml X-gal and 50 μg/ml hygromycin. Colonies were grown in 7H9 and double recombinants were selected for 7H11 agar with X-gal and 10% sucrose (w/v). White colonies were verified for Kan^S and Hyg^S and *lsr2* was sequenced

to confirm that allelic replacement had occurred. One colony was saved and named strain DL2008.

Colony morphology analyses and motility assays. For colony morphology analyses, *M. smegmatis* strains were grown on Middlebrook 7H11 (Difco) agar medium. Motility assays were performed as described previously (22). Briefly, cells were cultured in 7H9 medium to mid-logarithmic phase (OD_{600} 0.8-1.0) before spotting 2-μl aliquots onto motility medium consisting of M63 salts liquid medium (Difco) supplemented with 0.5% casamino acids, 0.2% glycerol, 1 mM $MgCl_2$, and 10 μM $FeCl_2$ and solidified with agarose. The agarose concentrations were either 0.3% or 0.8% (w/v). The inoculated plates were incubated for 24 h at 37°C in plastic bags containing moistened paper towels to ensure bacteria grew under humidified conditions.

Cell clumping, pellicle formation, and biofilm assay. The ability of *M. smegmatis* strains to clump in culture was analyzed in both 7H9 and M63 liquid media. Cells were grown to mid-log phase in medium with or without 0.05% Tween 80 in a 37°C shaker incubator. Cultures then sat at room temperature (RT) for 1 h to allow cell aggregates to settle. Pellicle formation was monitored, as previously described (6), using standing cultures with 0.05% Tween 80 at 37°C for 48 h. Biofilm formations on the sides of the wells in polyvinylchloride (PVC) and polystyrene microwell plates were performed as previously described (6, 31, 32).

Time-lapse imaging of spreading motility. Time-lapse photography was performed on 2-µl aliquots of mid log-phase cultures spotted onto M63 medium with 0.3% agarose. Plates were incubated at 37°C in a chamber with constant humidity, and images were taken every 10 min for 48 h with a color CCD camera (Jai Corporation, model CV-S3200). Video output from the camera was digitized with a DVBridge (Dazzle Inc). Images were captured at 10-min intervals with a Macintosh computer (iMac) using QuicktimePro 5.0.

Antibiotic sensitivity assays. The wild-type mc^2155 strain and the *lsr2*-mutant DL2008 strain were transformed with an integrative plasmid (pMV306) containing a single copy of the kanamycin resistance gene (*aph*). Paper discs, soaked in kanamycin sulfate ranging from 0-1.5 mg, were placed onto 7H11 plates swabbed with bacteria (grown in 7H9 + 0.05% Tween 80 to OD_{600} 0.1) and then allowed to incubate at 37°C. Zones of inhibition were observed and their diameters measured after 48 h of incubation at 37°C.

RT-PCR analysis. Relative levels of *aph* expression in different *M. smegmatis* strains was performed by limiting-dilution reverse transcription (RT)-PCR analysis, as previously described (4). The forward primer for 16s rRNA is 5'-CCGCAAGGCTAAAACTCAAA-3' and the reverse primer for 16s rRNA is 5'-TAACAAGGTAGCCGTACCGG-3'. The forward primer for *aph* is 5'-GGGAAAGCCACGTTGTGT-3' and the reverse primer for *aph* is 5'-AGGTCTGCCTCGTGAAGAAG-3'.

Plaque assays. Infections of *M. smegmatis* strains were carried out with the mycobacteriophage phAE159, as described previously (2). Briefly, 10-ml bacterial cultures were grown to mid-log phase (OD_{600} 0.8), washed twice in MP buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 24 mM $MgCl_2$, 2 mM $CaCl_2$) supplemented with 5% glycerol, resuspended in $1/10^{th}$ volume of MP buffer without glycerol, and then mixed with phage at a ratio of 1 x 10^7 bacteria: 15 phage. Following incubation at 30°C for 2 h, the phage-bacteria mixtures were added to top agar (0.3% agar w/v) and poured onto 7H11 plates. Resulting plaques were counted and photographed after incubation at 30°C for 48 h.

Electron microscopy. For TEM analysis of whole cells growing on M63 + 0.3% agarose, cells were lifted off of plates and stained, as previously described (22). Briefly, formvar carbon-coated nickel grids were pressed gently onto the surface of colonies and allowed to absorb material for 3-5 sec before removing and rinsing twice with dH_2O by floating grids sample-side down on 100-µl droplets. Samples were then stained either with 1% uranyl acetate (UA), 2% phosphotungstic acid (PTA) (pH 7.5), or 1% ammonium molybdate (AM) for 1 min before rinsing grids again with dH_2O. Samples were then viewed using a JEOL TEM 1200 EX, (JEOL USA, Inc. Peabody, MA). Images were acquired using Soft Imaging Systems (SIS) Analysis 3.5 Imaging software and a MegaView III camera (Lakewood, CO). For TEM analysis of cell envelopes, cells were grown in liquid 7H9 medium with 0.05% Tween 80 and then prepared and visualized as previously described (8).

Preparation of lollipop-shaped structures from culture supernatants.

Cultures were grown in M63 liquid with 0.05% Tween 80 to OD_{600} 1.5 before subjecting to the warring-blender action of a FastPrep FP120 device (Thermo Savant) set at 6.5 for 45 sec without glass beads present. Cultures were then centrifuged twice at 10,000 x g for 10 min to remove whole cells and large debris. Culture supernatants were then centrifuged at 50,000 x g for 3 h. All centrifugations took place at 4^{o}C. Pellets were resuspended in 1/100 volume M63 + 0.05% Tween 80 and 2-µl aliquots were dried onto formvar-coated grids before staining with 2% PTA for 5 min, washing grids by floating on water droplets, and viewing with TEM.

RESULTS

Selection of the DL1215 mutant. During the original competitive starvation assays between *M. smegmatis* mc^{2}155 and Δ*rel*$_{Msm}$, the two strains were mixed together in equal concentrations and subjected to three different stress conditions: resuspension in Tris-buffered saline, gradual growth into stationary phase in 7H9 + 0.05% Tween 80, or gradual depletion of oxygen in stationary 7H9 + 0.05% Tween 80 cultures (9). At various time points, the mixtures of cells were serially diluted onto 7H11 agar with hygromycin (to select for mc^{2}155 cells containing a Hyg^R cassette) or with kanamycin (to select for *M. smegmatis* Δ*rel*$_{Msm}$ cells containing a Kan^R cassette inside the *rel*$_{Msm}$ gene). After three weeks of competitive starvation under all conditions, the kanamycin-resistant cells resulted in two different colony morphologies. One type was wrinkled, dry, nippled, and resembled the original *M. smegmatis* Δ*rel*$_{Msm}$ strain previously described (9). The second colony morphology was flat, shiny and mucoid in appearance. This flat, shiny phenotype was never seen in the wild-type mc^{2}155 cells starved for equal lengths of time. This spontaneously appearing, shiny mutant was named DL1215 and when grown for more than three weeks on 7H11 agar, DL1215 developed a perimeter with ruffled edges (Fig. 1A). Growth rates of *M. smegmatis* mc^{2}155, Δ*rel*$_{Msm}$, and DL1215 in 7H9 + 0.05% Tween 80 liquid medium are identical (data not shown). 16s rRNA sequencing confirmed that DL1215 is a strain of *M. smegmatis* (data not shown).

Complementation of the DL1215 strain with *lsr2* gene. Chen *et al.* recently reported that a transposon insertion into the *lsr2* gene of *M. smegmatis* mc^{2}155 results in

a *M. smegmatis* strain (MS8444 mutant) with smooth colonies similar in appearance to strain DL1215 (6). To test if the DL1215 mutant reported here contains a defect in its *lsr2* gene, the strain was transformed with pLSR2 (an *E. coli-Mycobacterium* shuttle vector containing a wild-type copy of *lsr2* under its native promoter) (Table 1). The resulting strain, DL1215/pLSR2, formed rough-looking colonies resembling the parental *M. smegmatis* Δrel_{Msm} strain (data not shown). When DL1215 was transformed with the cloning vector alone (pNBV1), however, there was no alteration in colony morphology. Sequence analysis of *lsr2* confirmed that strain DL1215 contains a mutation in this gene. Loss of a single adenine nucleotide at the 40th codon resulted in a frame-shift mutation leading to a stop codon eight codons downstream (Fig. 1B). The DL1215 Lsr2 protein was <42% the length of the protein from the *M. smegmatis* mc^{2}155 and Δrel_{Msm} strains. We believe that even though DL1215 is capable of producing a truncated version of Lsr2 protein, this strain acts as though it has a null mutation for *lsr2* since it is so similar in colony morphology (Fig. 1A) and hydrophobicity (Fig. 2) for what was reported previously for the MS8444 mutant containing a transposon insertion in the *lsr2* gene.

Inactivation of *lsr2* in mc^{2}155 by allelic replacement. Because strain DL1215 has both inactivated rel_{Msm} and *lsr2* genes, it is not clear which unique DL1215 phenotypes are due to just *lsr2* inactivation and which might be due to the double mutation. Also, because DL1215 arose spontaneously from the Δrel_{Msm} strain it is possible DL1215 has more than one unmarked mutation. To address both of these points we replaced the *lsr2* allele in mc^{2}155 with the mutant *lsr2* allele from DL1215 to create

strain DL2008. DL2008 has a functional rel_{Msm} gene and yet it displayed the same smooth-colony phenotype as that of DL1215 (data not shown).

Analysis of cell surface hydrophobicities and of pellicle and biofilm formations. Since MS8444 and DL2008 both have dysfunctional *lsr2* genes, we examined the hydrophobicity of DL2008 as had previously been done for MS8444. Usually a detergent like Tween 80 (0.05% v/v) is required for growth of mycobacteria as dispersed cells in liquid cultures (Fig. 2A, all tubes). Growth in liquid medium without Tween 80 results in cell aggregation that leads to reduced turbidity in shaking cultures of mc^2155 and *M. smegmatis* Δrel_{Msm} (compare tubes 1 and 2 in Fig. 2A and 2B). However, the DL2008 and DL2008/pNBV1 strains remain completely dispersed in culture even without Tween 80 (Fig. 2B; tubes 3 and 5), while DL2008/pLSR2 resembles the wild-type and Δrel_{Msm} strains in regards to clumping (Fig. 2B; tube 4). Dispersion of DL2008 and DL2008/pNBV1 cultures without Tween 80 is likely due to reduced surface hydrophobicities of these cells.

Chen *et al.* have also shown the requirement of Lsr2 in pellicle formation at the air-liquid interface of stationary 7H9 liquid cultures (6). The DL1215 mutant was similarly tested in standing cultures of both M63 + 0.05% Tween 80 (Fig. 2C) and 7H9 + 0.05% Tween 80 (data not shown). In both media, DL2008 and DL2008/pNBV1 were not able to form thick pellicles above the liquid surfaces (Fig. 2C; tubes 3 and 5) although mc^2155, Δrel_{Msm}, and DL2008/pLSR2 strains did form visible surface pellicles above the air-liquid interface (Fig. 2C; tubes 1, 2, and 4).

The lack of ability to form pellicles correlates with defect in biofilm formation (6). Therefore, the ability to form biofilms on polyvinylchloride (PVC) and polystyrene plastic surfaces was also tested. Figure 2D shows that both DL2008 and DL2008/pNBV1 did not appreciably adhere to the sides of polystyrene tissue-culture wells and consequently these wells did not stain with crystal violet. These strains also did not adhere to PVC wells (data not shown). This is in contrast to the other *M. smegmatis* strains tested which were capable of forming biofilms that stained with crystal violet. Therefore, our results confirm the previously reported role of Lsr2 in regulating these cell surface-associated phenotypes. The requirement of Lsr2 for biofilm formation was further confirmed by performing floating biofilm assays in plastic petri dishes, as described previously (25) (data not shown).

Cell motility on plates with low concentrations of agarose. A phenotype not reported for MS8444 is its surface sliding ability. Previously, Roberto Kolter's laboratory has shown that mc^2155 is motile on media with 0.3% agarose, and that this motility was dependent upon the ability of *M. smegmatis* to produce and acetylate GPLs (31, 32). Based upon the observation of the smooth-colony nature of the *lsr2* mutant (Fig. 1) (6), we compared its motility to that of other isogenic strains (Fig. 3A). Two-µl aliquots of mid log-phase cultures (OD_{600} 1.0) were spotted onto M63 plates with 0.8% or 0.3% agarose (w/v). At 0.8% agarose, all colonies are intact and have a smooth circumference (Fig. 3A1, 3A3, and 3A5) while at 0.3% agarose DL2008/pNBV1 lacks the defined colony edges seen for the other strains (Fig. 3A4). The edges of *M. smegmatis* mc^2155 and DL2008/pLSR2 colonies show that cells are all adjacent to one

another (Fig. 3A2 and 3A6, respectively). However, the DL2008/pNBV1 colony edge appears completely fragmented with groups of cells migrating as independent "rafts" apart from one another (Fig. 3A4). These "rafts" of DL2008/pNBV1 cells appear to be floating in a layer of slime. When a light source is positioned at an angle to a DL2008 colony on M63 + 0.3% agarose, a fragmenting pattern of cells is seen (Fig. 3B; arrows) in addition to the light source reflecting off the edge of a shiny river of slime extending beyond the edges of the colony (Fig. 3B; arrowheads). Figures 3C and 3D show colony edges of mc^2155 and DL2008 strains, respectively, on M63 plates with 0.3% agarose. Whereas mc^2155 cells grow on top of each other and the colony edge is smooth (Fig. 3C), DL2008 cells exist in a monolayer and aggregates of cells are seen dispersed from the main colony on the solid medium (Fig. 3D).

Time-lapse photography of the mc^2155, *M. smegmatis* Δrel_{Msm}, and the DL1215 strains migrating on the same M63 + 0.3% agarose plate is shown in figure 4A. Over a 40-h period the DL1215 shows motility at the rate of 31.25 μm/min, which is faster than mc^2155 or Δrel_{Msm} growing on the same plate (see Quick Time movie in online supplementary data). Strain DL2008 shows this same level of "hyper-motility" as DL1215, and complementation of DL2008 with *lsr2* results in a loss of this excessive motility (Fig. 4B). These results indicate that Lsr2 plays a role in regulating surface spreading in *M. smegmatis*. Interestingly, this effect of Lsr2 is influenced by the stage of growth. The excessive motility of DL1215 and DL2008 is exhibited by mid-log phase cells whereas stationary-phase cultures (3-days old) spotted onto M63 + 0.3% agarose do not show any migration in excess of mc^2155 (data not shown).

Extracellular structures detected by transmission electron microscopy of whole cells. In order to better observe cells at the periphery of the motile mass, TEM analysis was performed. Whole cells were lifted from the surfaces of M63 + 0.3% agarose plates near the edges of spreading colonies (Fig. 5). The mc^2155 cells at the periphery of colonies had PTA-stained, extracellular material associated with them, as has been previously described (Fig. 5A; arrow) (22). This dark extracellular staining was also evident for numerous DL1215 cells lifted from plates (Fig. 5B; solid arrow). However, in addition to this darkly-staining material, the DL1215 cells also had diffuse matrices surrounding them (Fig. 5B; open arrow). These diffuse matrices from several different cells were capable of joining together to form a connective layer (Fig. 5C; arrow). When viewed under higher magnifications, these layers seemed to be comprised of negatively-stained structures resembling bacteriophage heads connected to tails (Fig. 5D, 5E, and 5F). These "lollipop" structures were only visible in the DL1215 and DL2008 cells (Fig. 5G) and only when these cells were stained with PTA. UA and AM staining did not allow for visualization of these structures (data not shown). These structures were absent from mc^2155, Δrel_{Msm}, DL1215/pLSR2 cells, and DL2008/pLSR2 (data not shown). Such extra-cellular structures were not reported by Chen *et al.* who first described a *lsr2* mutation in *M. smegmatis* (6). Formvar-coated grids used to lift material from areas of the plate containing light-refractive slime but no visible cells (see Fig. 3B) did not have any PTA-staining material (data not shown). When mc^2155, Δrel_{Msm}, and DL2008/pNBV1, and DL2008/pLSR2 cells were grown in M63 liquid with 0.05% Tween 80, "lollipop" structures were only observed in the culture supernatant of DL2008/pNBV1 and not from any of the other three strains (data not shown).

Taken together, these finding indicate that Lsr2 has a role in regulating the production of these extracellular structures from *M. smegmatis* cells. It cannot be said with certainty if these structures are involved in spreading of *M. smegmatis* on soft agar surfaces. However, the appearance of these structures in association with the hyper-motile strains DL1215 and DL2008 suggests that they may represent an as yet unidentified mechanism of mycobacterial motility.

Loss of Lsr2 increases resistance to mycobacteriophages. Loss of hydrophobicity in DL2008 raises the possibility of an alteration in cell envelope permeability. Parish *et al.* have previously characterized a *M. smegmatis* mutant with increased hydrophobicity and altered cell envelope permeability. This mutant was initially characterized as producing unusually large plaques when infected with various mycobacteriophages (27). Based upon this reported link between cell hydrophobicity, cell permeability, and phage infection, the *M. smegmatis* strains in this study were tested for infection by the general transducing mycobacteriophage phAE159 (Fig. 6) and phAE86 (data not shown). The mc^2155, Δrel_{Msm}, and DL2008/pLSR2 strains all produced comparable numbers of plaques (Fig. 6A). However, phAE159 infections of DL2008 and DL2008/pNBV1 produced fewer numbers of plaques that were also more turbid. Appearances of phAE159 plaques on lawns of DL2008/pLSR2 and DL2008/pNBV1 are shown in figures 6B and 6C, respectively. The reduced number of plaques for DL2008 and DL2008/pNBV1 could be due either to reduced entry/replication of phage in the host cells or to reduced attachment on the host cell surfaces. To test this hypothesis, TEM analysis was performed on whole cells allowed to adsorb phage. No

difference was seen between the average numbers of visibly-bound phage to cell surfaces for *M. smegmatis* strains with or without functional *lsr2* (data not shown). Therefore, we believe the reduced numbers of plaques in DL2008 and DL2008/pNBV1 were due to a reduction in phage entering or replicating within the host cells indicating that Lsr2 has a role in regulating susceptibility to mycobacteriophage.

Lsr2 affects sensitivity to kanamycin. DL2008 was tested for susceptibility to a variety of antibiotics. Strains mc^{2}155, Δrel_{Msm}, and DL2008 showed identical susceptibilities (diameters of zones of inhibition) to discs containing the following amounts of antibiotics: 10 μg penicillin, 1 μg oxacillin, 15 μg erythromycin, 30 μg cephalothicin, 30 μg vancomycin, 100 μg carbenicillin, 10 μg gentamicin, 30 μg amikacin, 30 μg amoxicillin/clavulanic acid, 5 μg ciprofloxacin, and 23.7 μg sulfamethoxasole/1.25 μg trimethoprim (data not shown). The three strains also showed no differences in suscepibilities to discs soaked with 1-1500 ng of rifampin (data not shown).

One antibiotic susceptibility pattern not reported for the MS8444 strain was for kanamycin sulfate. The original *M. smegmatis* Δrel_{Msm} strain had a kanamycin-resistance cassette inserted into its rel_{Msm} gene (9). The spontaneous mutant DL1215 that arose from this had a greatly enhanced kanamycin-resistance compared to the Δrel_{Msm} strain (data not shown). It was therefore hypothesized that Lsr2 played a role in resistance to this antibiotic. The wild-type strain mc^{2}155 and the *lsr2*-mutant strain DL2008 were made kanamycin resistant by transforming cells with an integrative plasmid (pMV306) carrying the Kan^R marker. Although they contain the kanamycin-resistance cassette (*aph*

gene) on the same position on the chromosome (insertion at the *attB* site), DL2008::pMV306(K) showed much greater resistance than the *M. smegmatis* mc^2155::pMV306(K) parental strain (Fig. 7A). DL2008::pMV306(K)/pLSR2 zones of kanamycin inhibition resembled those of mc^2155::pMV306(K) (Fig. 7A). Since the DL2008::pMV306(K)/pNBV1 strain showed no difference in kanamycin resistance from DL2008::pMV306(K), the *lrs2* gene clearly plays a role in altering kanamycin susceptibility of this strain. RT-PCR analysis shows that the level of *aph* expression is higher in DL2008::pMV306(K) than in the parental mc^2155::pMV306(K) strain (Fig. 7C). This suggests Lsr2 exhibits some direct or indirect repression of *aph* expression.

Plasmid replication requires Lsr2. Without antibiotic selective pressure, episomes were lost quickly from the replicating *M. smegmatis* strain missing a functional *lsr2* gene (Fig. 8). The pool of liquid-grown DL2008/pNBV1 cells lost its ability to grow on 7H10 agar plates with hygromycin (50 μg/ml) after only a few passages without selective pressure. However, *M. smegmatis* mc^2155/pNBV1, mc^2155/pLSR2, and DL2008/pLSR2 all showed only a modest loss of plasmid DNA after multiple passages in 7H9 liquid medium without hygromycin. These results suggest *lsr2* may play some role in plasmid replication or plasmid segregation to daughter cells. We do not believe Lsr2 plays a significant role in chromosomal DNA replication since we observe DL2008 to grow at the same rate as mc^2155 cells in liquid culture (data not shown).

Analysis of cell envelope components. It has been reported that production of GPLs is needed for *M. smegmatis* motility (22, 31, 32). We analyzed GPL content for

our *lsr2* proficient and deficient strains. DL1215 is identical with its parental strain (Δrel_{Msm}) for GPL content (online supplementary data). This is not surprising because the previously reported *lsr2*-mtuant strain, MS8444, also did not show any difference in its GPL profile (6) Therefore, our findings show that DL1215 hyper-motility (Fig. 4A) is not due to alteration of GPL content. In addition to GPL content, we discovered the mycolic acid compositions and polar lipid compositions of mc^2155, Δrel_{Msm}, and DL1215 to be identical (data not shown). This is also in agreement with the MS8444 mutant (6). However, MS8444 was shown to lack two apolar lipids identified as novel mycolate compounds (6). Two-dimensional TLC analysis of DL1215 apolar lipids in this current study failed to show any difference from the profiles of mc^2155 and Δrel_{Msm} (data not shown). Calcofluor white was used to stain surface-exposed carbohydrates of mc^2155, Δrel_{Msm}, and DL1215, but no differences were observed for these three strains (data not shown). As with the MS8444 mutant (6), TEM analyses of cell wall ultrastructures did not reveal any novel features in the DL1215 cell envelope (data not shown). Collectively, analysis of the cell envelope failed to reveal a structural or molecular explanation for the DL1215 colony morphology, cell hydrophobicity, or hyper-motility.

Elevated levels of ribosomal protein L22 in *lsr2*-deficient strains. Although molecular analysis of cell envelopes revealed no differences between the Δrel_{Msm} and DL1215 strains for GPLs, mycolic acids, polar lipids, and apolar lipids, this was not true for whole-cell protein comparisons. After growing mc2155, Δrel_{Msm}, and DL1215 strains in liquid 7H9 with 0.05% Tween 80 to mid-log phase, their protein compositions were determined by 1-D SDS PAGE. When equal amounts of protein from the lysates were

compared, the protein profiles of the different strains were nearly identical with the exception of a single species (online supplementary data). Elevated levels of a 17-kDa protein were present in DL1215 and DL1215/pNBV1 compared to mc^2155, Δrel_{Msm}, and DL1215/pLSR2. Peptide mass finger printing identified this protein as the L22 ribosomal protein. This result agrees with recently reported microarray analysis showing that the gene for L22 is up-regulated >2-fold in an *lsr2*-defiencient *M. smegmatis* strain compared to a WT strain (7).

DISCUSSION

This report describes a hyper-motile strain of *M. smegmatis* that spontaneously arose from *M. smegmatis* Δrel_{Msm} cells exposed to conditions of oxygen and nutrient deprivation (9). This strain, *M. smegmatis* DL1215, is the result of a mutation, rather than a phase variation. The nature of this mutation is a single base-pair deletion leading to a frame shift in the *lsr2* gene (Fig. 1B). The *lsr2* gene was subsequently inactivated in wild-type mc^2155 to create strain DL2008. Lsr2 is a basic, cytosolic protein with no known functional motifs, but it has been shown to interact with DNA and serve as a regulatory protein (7). Although the biological role of Lsr2 has not been elucidated yet, it is recognized as an immunodominant T-cell antigen in *M. leprae* (20, 24). The transposon-insertion inactivation of *lsr2* from *M. smegmatis* mc^2155 has previously been reported for strain MS8444 (6). MS8444 and DL2008 have numerous similarities in their morphological phenotypes. However, here we report phenotypes not discussed before with respect to Lsr2, which include enhanced resistance to kanamycin, hyper-motility, production of lollipop-shaped particles, and no change in apolar lipid profile in DL2008.

The *lsr2*-deficient strain DL2008::pMV306(K) demonstrates approximately 2.5-fold greater resistance to 250 μg of kanamycin than mc^2155::pMV306(K). This enhanced resistance could potentially be due to a number of different phenomena including: efflux of the antibiotic to lower the cytoplasmic concentration, elevated expression levels of the single *aph* gene in the cell, amplification of kanamycin targets, or decreased permeability of the cell envelope to the antibiotic. It is evident that Lsr2 has a role in regulating this effect since complementation of DL2008::pMV306(K) with pLSR2

abolishes the ability to grow in high concentrations of the antibiotic (Fig. 7A). Alteration in cell permeability to kanamycin is not likely based upon a recent report by Colangeli *et al.* showing that *M. smegmatis* inactivated for *lsr2* is not altered in cell permeability for hydrophobic or hydrophilic compounds (7). RT-PCR analysis indicates that enhanced kanamycin resistance is due to elevated *aph* expression in the absence of Lsr2 (Fig. 7B). Therefore, Lsr2 acts as either a direct or indirect repressor of *aph* expression.

Lsr2 has a distinct role in determining the cell envelope composition of *M. smegmatis*. As shown here and by Chen *et al.* (6), Lsr2 influences the ability of *M. smegmatis* to form biofilms, leads to the formation of smooth colonies, and alters the ability of *M. smegmatis* cells to clump in liquid culture. All of these are traits directly linked to hydrophobicity of mycobacterial cell walls. Biofilm and pellicle formation and aggregation of cells in culture have been shown to be altered by changes in cell surface hydrophobicities (6, 25). Therefore, it can be argued that Lsr2 inactivation leads to a reduction in the hydrophobicity of the DL1215 and DL2008 cell walls. In order to investigate this further, we performed biochemical analysis of cell envelope lipids of *lsr2*-deficient cells. It is not surprising that DL1215 shows no difference in its profiles for GPLs, polar lipids, and mycolic acids since the MS8444 strain also fails to show any differences in these classes of lipids (6). In contrast to MS8444, DL1215 does not show any difference in the profile of apolar lipids. This lack of difference in apolar lipids has been documented previously for another independent *lsr2* mutant as well (7).

The most interesting feature of the *lsr2*-inactivated strains is the enhanced motility ("hyper-motility) on solid surfaces (Fig. 4). GPL-dependent motility has previously been demonstrated for *M. smegmatis* mc^2155 (22, 31, 32). We observed a

modest amount of motility for mc^2155 in this study (Fig. 4A and movie in the online supplementary data). However, DL1215 exhibited upwards to a 12.5-fold greater motility rate (31.25 μm/min) than mc^2155 (1.6 μm/min) and the smooth colony mutant, Sm-1 (2.5 μm/min) described previously (22). Since the DL1215 mutant is not altered for GPL content (online supplementary data), we believe this hyper-motility involves additional cellular systems than the GPLs. These cellular systems might be negatively regulated by Lsr2. It is possible that the lollipop-shaped ultrastructures observed here, while appearing in hyper-motile strains, are not directly associated with the enhanced motility. Hyper-motile strains might result from a reduced amount of adhesion to the agar surface that leads to increased sliding.

Strain DL2008 produces smooth colonies characterized by increased mucoidy. Other mycobacterial species capable of generating smooth-colony morphotypes have been reported (1, 5, 18, 19, 28). The smooth-colony phenotype of DL2008 is likely to be linked to changes in the hydrophobicity of the cell wall (Fig. 2). However, an interesting speculation is that colony morphology might be linked to production of lollipop-shaped extra-cellular structures resembling phage particles (Fig. 5). Precedence exists in *M. smegmatis* for lysogeny causing a shift from rough to smooth colony morphology (11, 14, 15, 17, 21, 34), and it has been proposed that *M. smegmatis* is a polylysogen (11, 14, 33). The correlation of these phage-like structures with inactivation of *lsr2* suggests that absence of Lsr2 may induce lysogens. Mycobacteriophage proteins Gp39 and Gp61 (from phage Cjw1 and Omega, respectively) share some homology to Lsr2 suggesting the gene could have arisen in mycobacteria by horizontal gene transfer (29). Annotation of the *M. smegmatis* genome has not revealed the presence of prophages. However, this

absence of prophage identification could be due to the high degree of genetic diversity of mycobacteriophages and an inability to identify them by sequence gazing (29). The lollipop-shaped particles reported here have heads of varying diameter and tail length as well as filamentous particles lacking any head structures. The correlation of these extra-cellular structures with inactivation of *lsr2* suggests a possible link between their production and bacterial motility.

It has been reported that a gene encoding a type III restriction enzyme, MSMEG_1238, is 4.5-fold up regulated in the absence of Lsr2 (7). If this is true, then the MSMEG_1238 gene product can be expected to prevent foreign DNA from replicating in the bacterial cell. This may explain why DL2008 is more resistant to phage infection and subsequent plaque formation (Fig. 6). An alternative explanation for reduced plaque formation in DL2008 is that Lsr2 is a host-cell protein needed for efficient phage replication.

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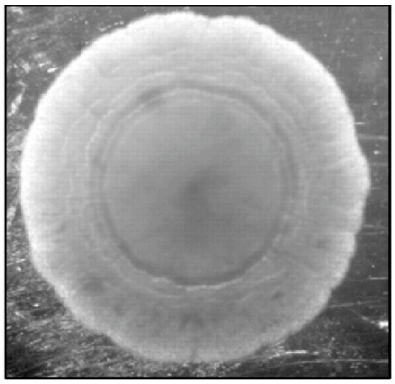
TABLE 1. Bacterial strains and plasmids.

Bacterial strains	characteristics	reference
STRAINS		
M. smegmatis mc^2155	wild-type strain	38
M. smegmatis Δrel_{Msm}	no stringent response; Kan^R	9
M. smegmatis::pMV306(K)	wild-type strain; Kan^R	this study
DL1215	spontaneous mutant appearing from *M. smegmatis* Δrel_{Msm}; enhanced Kan^R	this study
DL1215/pNBV1	Hyg^R	this study
DL1215/pLSR2	extrachromosomal copy of *lsr2*; Hyg^R	this study
DL2008	*M. smegmatis* with unmarked *lsr2* mutation	this study
DL2008/pNBV1	Hyg^R	this study
DL2008/pLSR2	extrachromosomal copy of *lsr2*; Hyg^R	this study
DL2008::pMV306(K)	*lsr2* mutant strain; Kan^R	this study
DL2008::pMV306(K)/pNBV1	Kan^R; Hyg^R	this study
DL2008::pMV306(K)/pLSR2	Kan^R; extrachromosomal copy of *lsr2*; Hyg^R	this study
PLASMIDS		
pNBV1	cloning shuttle vector; Hyg^R	13
pLSR2	*lsr2* gene cloned into pNBV1	6
pMV306	mycobacterial integrative vector; Kan^R	39
pDrive	cloning vector for PCR products	Qiagen
pGOAL19	plasmid with Hyg^R, *lacZ*, and *sacB* cassette	26
p2NIL	plasmid for allelic replacement; Kan^R	26
pKA0505	*ls2* gene from strain DL1215 cloned into p2NIL with pGOAL19 marker cassette	this study

BACTERIOPHAGE

phAE159	general transducing mycobacteriophage	2

A



B

```
mc²155:   MAKKVTVTLVDDFDGEATADETVEFGLDGVTYEIDLSAKNAAKLRNDLKQWVEAGRR 57
DL1215:   MAKKVTVTLVDDFDGEATADETVEFGLDGVTYEIDLSAKTPRSCATI********** 47

mc²155:   VGGRKRGRAATTTTRGRGAIDREQSAAIREWARRNGHNVSTRGRIPADVIDAFHAAT 114
DL1215:   ************************************************************
```

Figure 1. The *lsr2* gene is required for normal *M. smegmatis* colony morphology. (A) 7H11 agar plate with a single DL1215 colony grown for 2 weeks. Colony is flat and smooth compared to parental Δrel_{Msm} colony (see Fig. 2 in ref. 9). (B) Sequence alignment of Lsr2 from mc^{2}155 (WT) and DL1215 strains of *M. smegmatis*. The underlined sequence indicates residue changes from WT Lsr2 protein and asterisks show residues missing in the mutant protein.

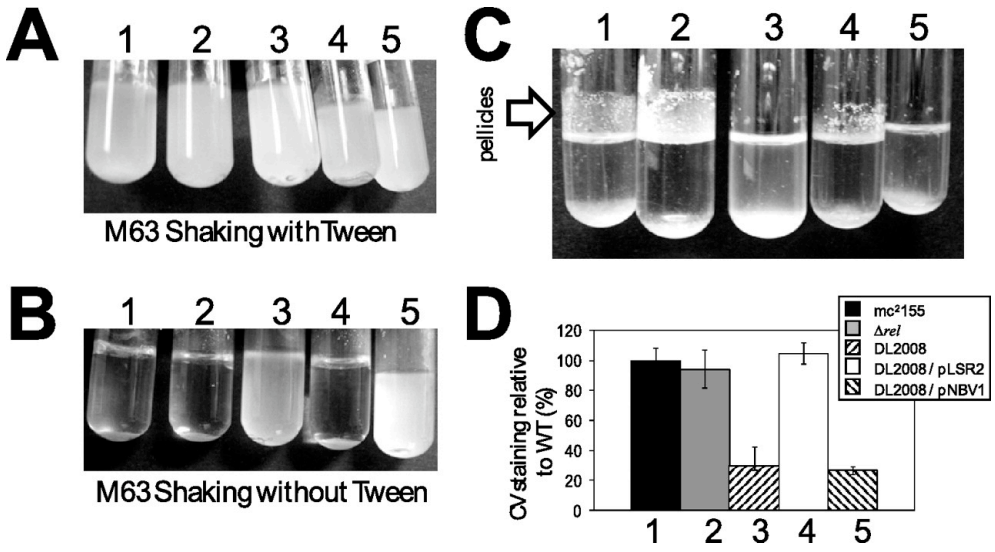


Figure 2. Lsr2 is required for biofilm formation. Strains include 1, mc^2155; 2, Δrel_{Msm}; 3, DL2008; 4, DL2008/pLSR2; and 5, DL2008/pNBV1. When *lsr2* is functional, strains are very hydrophobic, as evident by their ability to stay in suspension when Tween 80 is present (A), but clump in the absence of Tween (B). Strains without *lsr2* function remain suspended without Tween. Pellicle formation was allowed to occur in standing cultures without Tween (C). Pellicles are missing if *lsr2* is inactivated. Biofilm formation occurred in polystyrene tissue culture wells if *lsr2* was functional (D). Crystal violet (CV) staining is shown relative to the WT (100%). Error bars indicate standard deviations for 12 separate wells per strain.

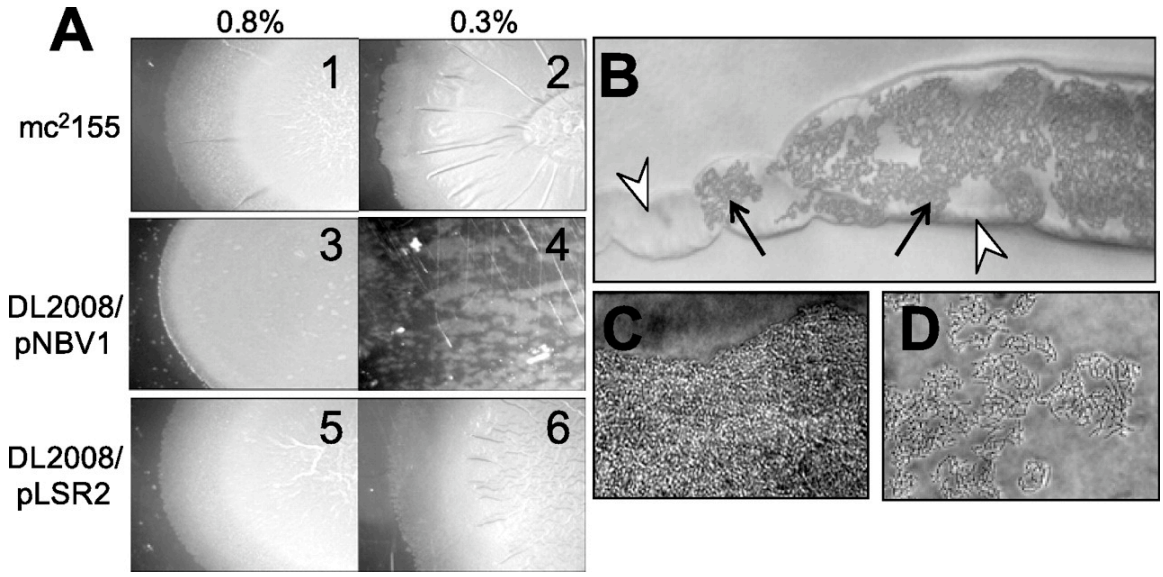


Figure 3. ***M. smegmatis*** **strains grown on low-agarose plates**. (A) The effect of agarose concentration on colony morphology is shown. 7H9 plates with decreased concentrations of agarose (0.8% and 0.3% w/v) were inoculated with 2-µl aliquots of *M. smegmatis* cultures grown to OD_{600} 1.0. After 7-days growth on plates, the edges of colonies were compared. On 0.3% agarose plates the DL2008 colony showed fragmentation with separate large patches of cells (panel A4). (B) DL2008 forms a layer of "slime" on M63 + 0.3% agarose, as visualized with a dissection microscope. The light refractive edge of the slime is shown (arrowheads) with patches of cells floating in it (arrows). Phase-contrast light microscope images of mc^2155 cells (C) and DL2008 cells (D) at the perimeter of colonies. Magnification 200X.

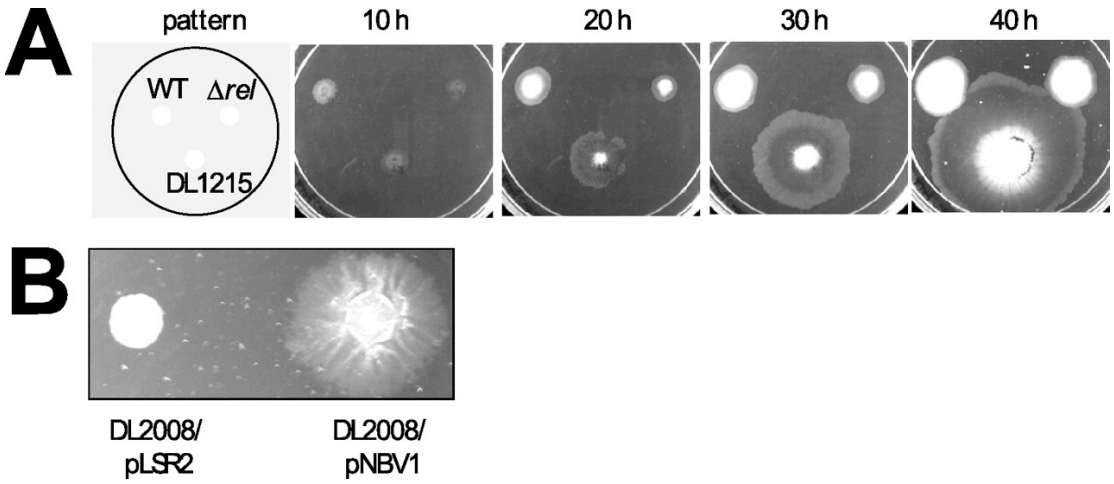


Figure 4. *M. smegmatis* strains inactivated for *lsr2* have a hyper-motility phenotype. (A) Several snapshots are shown for a 40-h period of a DL1215 colony migrating on M63 + 0.3% agarose. Two-µl aliquots of cells grown to OD_{600} 1.0 were spotted onto a humidified plate and allowed to grow. Relative migration of DL1215 is shown on the same plate with wild-type mc^2155 and Δrel_{Msm} strains. (B) Hyper-motility of DL2008 cells is eliminated by complementing the strain with the *lsr2* gene (DL2008/pLSR2). Motility of DL2008 is unaltered by the vector alone (pNBV1).

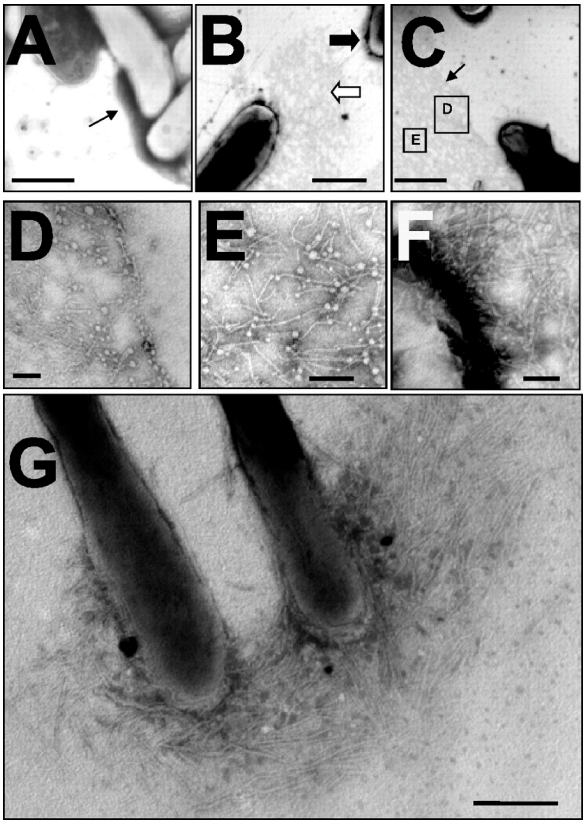


Figure 5. TEM analysis of PTA-stained *M. smegmatis* cells adhering to formvar-coated grids. (A) WT *M. smegmatis* mc^2155 growing on M63 + 0.3% agarose plates show the characteristic PTA-staining dark halos around the cell (arrow). (B) In addition to darkly-stained halos near the bacterial surface (closed arrow), DL1215 cells also have a diffuse, cloudy halo extending far from their surfaces (open arrow). (C) When DL1215 cells are close together, their diffuse extracellular halos can merge together to form a large area with a distinct boundary (arrow). The inserts in panel C are magnified in panels D and E. The edge of this diffuse halo (D) and an interior region of this halo (E) both show discrete structures resembling negative-stained rods with swellings at many of the ends ("lollipops"). (F) The edge of a PTA-stained DL1215 (lower left corner) cell shows these negative-stained structures extruding from the cell surfaces. (G) Extrusion of these negative-stained "lollipop" structures occurs from the poles in many cells and shown here for two parallel DL2008 cells on 0.3% agarose plates. Bars = 1 μm for panels A-C, 100 nm for panels D-F, and 0.5 μm for panel G.

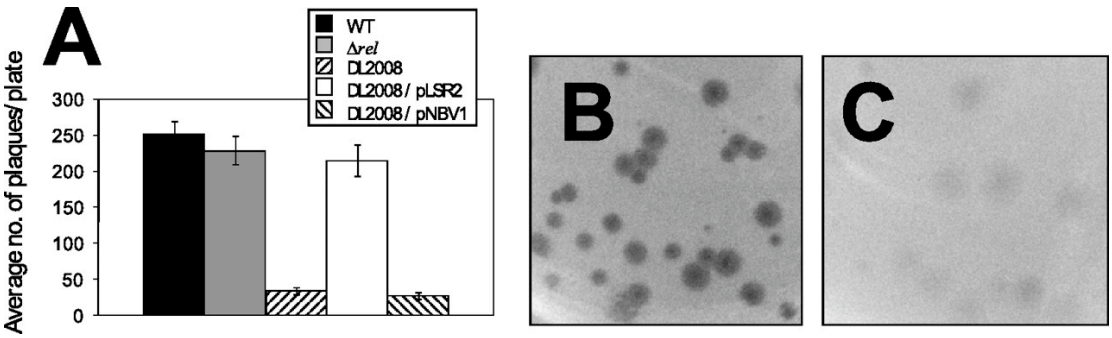


Figure 6. Inactivation of *lsr2* is associated with resistance to bacteriophage lysis. The general-transducing phage phAE159 was used to infect different *M. smegmatis* strains and then plaque numbers were scored. Inactivation of *lsr2* leads to a >4-fold decrease in susceptibility to phage infection (A). Presence of functional Lsr2 results in clear plaques in strain DL2008/pLSR2 (B) while absence results in turbid plaques in strain DL2008/pNBV1 (C).

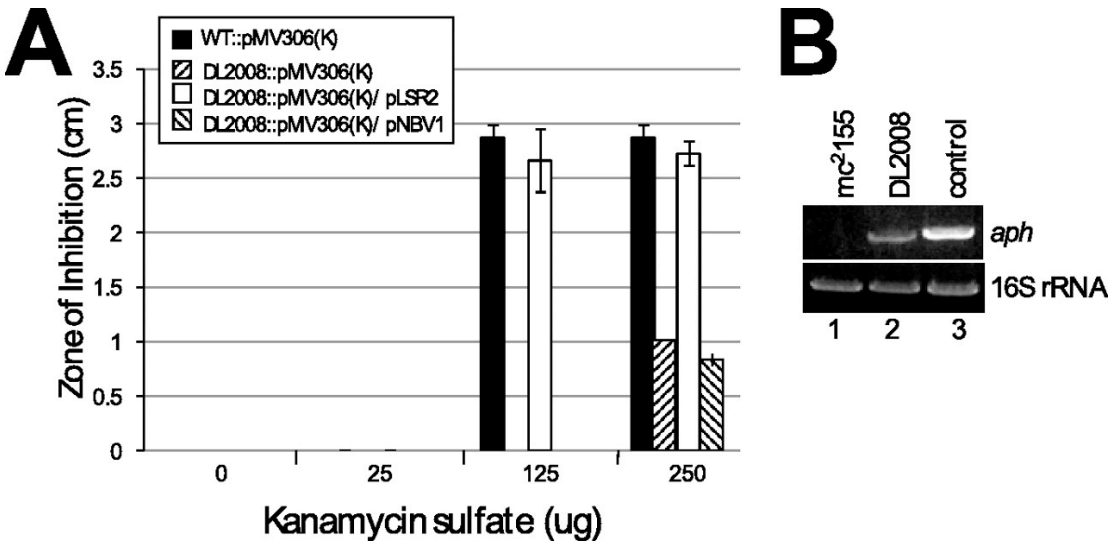


Figure 7. Lsr2 is associated with susceptibility to kanamycin. Zones of inhibition (cm) were measured around paper discs soaked with increasing amounts of kanamycin sulfate (A). Error bars represent standard deviations for 3 discs per antibiotic concentration. (B) Limiting-dilution RT-PCR analysis shows expression levels of *aph*, the gene responsible for Kan^R, are higher in the *lsr2* mutant background (DL2008) than in the parental mc^2155strain. Lanes 1 and 2 had cDNA as templates and lane 3 had DNA as a template. RT-PCR analysis of 16S rRNA was performed to ensure equivalent amounts of cDNA were used as template for PCR reactions. DNase-treated samples produced no PCR products (data not shown).

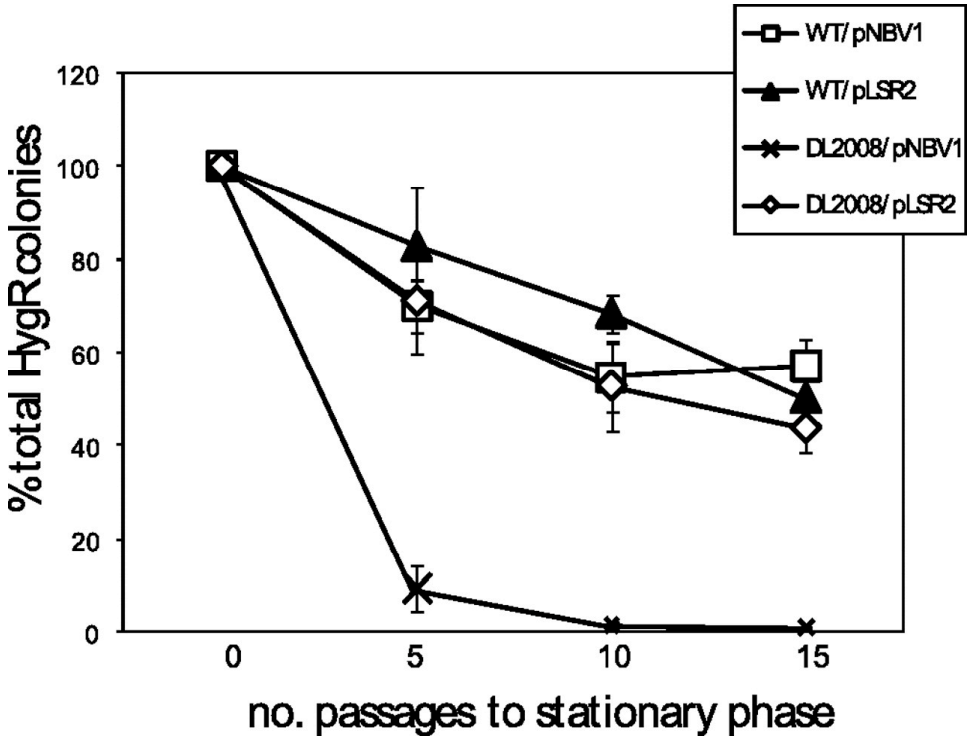


Figure 8. Maintaining plasmid DNA without selective pressure is dependent upon functional Lsr2. The extra-chromosomal plasmids pNBV1 (vector) and pLSR2 both contain hygromycin-resistance markers. When cells are repeatedly grown to stationary phase in the absence of antibiotic, hygromycin resistance is lost slowly if *lsr2* is present either on the chromosome (mc^2155) or on the plasmid (pLSR2). However, without a functional copy of *lsr2* (DL2008/pNBV1; open squares), hygromycin resistance is lost very quickly.

CHAPTER FIVE

Discussion

The work described in this dissertation has been aimed at addressing two questions. First, do the major pathogenic mycobacterial species *M. tuberculosis* and *M. bovis* differ in the ability to survive stress *in vitro?* Second, what is the role of the histone-like protein Hup in the physiology and survival of *M. smegmatis* in stressed and non-stressed conditions? The significance of this work centers on its contribution to the study of stress survival in pathogenic and nonpathogenic *Mycobacterium*. In many regards *Mycobacterium* are particularly hardy organisms. Some species are able to survive decades within a host, withstand desiccation and UV radiation, and endure drastic shifts in temperature. The ability to survive these potentially lethal conditions is one of the reasons *Mycobacterium* infections are so widespread, with an estimated one third of the world's population infected with a member of the tuberculosis complex.

Many of the genes in the regions of DNA missing from *M. bovis*, and many of the genes differentially regulated between *M. bovis* and *M. tuberculosis* are involved in cell wall biosynthesis. TLC analysis in chapter two supports the hypothesis that the cell walls of *M. bovis* and *M. tuberculosis* differ. As the cell wall is a major barrier against the environment, these differences in cell wall composition could account for the heightened stress resistance of *M. bovis* compared to *M. tuberculosis*. One additional change to the cell wall that the comparative SDS-PAGE of *M. tuberculosis* versus *M. bovis* showed in chapter two, was an increase in the level of HupB in the cell wall of *M. bovis.* HupB is both a cell wall associated and cytosolic protein that has been linked to the adhesion of *Mycobacterium* to mammalian cells (5, 9). Altered levels of HupB between *M. bovis* and *M. tuberculosis* could therefore affect the adhesion of both species to host cells. To examine this possibility, a cell adhesion assay could be performed. The

increase of HupB in *M. bovis* could lead to an increase in cellular attachment. However, even if a difference in cellular attachment is seen between *M. tuberculosis* and *M. bovis* this difference could not be definitively linked to the level of HupB. The many differences in the cell wall lipids could account for any potential differences in cellular attachment during infection of the host. One way to try and address the importance of HupB in cellular attachment would be to create a *hupB* knockdown in *M. bovis*. If HupB were involved in attachment to host cells, it would be expected that the knockdown would have decreased cellular adhesion.

One of the first phenotypic differences detected in the *M. smegmatis* Δ*hup* strain was the colony morphology. This change in morphology suggested changes in the cell wall that were also apparent in the lack of cellular aggregation in liquid media without Tween 80. Possible reasons for a smoother colony and ability to grow dispersed in liquid could be modifications to the lipids that make up the cell wall. To test this idea, thin-layer chromatography (TLC) was performed on cell wall components extracted from the *M. smegmatis* wild-type and Δ*hup* cell walls (data not shown). As no difference was detected using this technique, a more sensitive TLC detection technique could be utilized involving C^{14} labeling of cell wall lipids (1). This would allow more sensitive detection of cell wall differences. Mass spectroscopy of spots differentially expressed would allow identification of the specific lipids that differ from *M. smegmatis* wild-type to *M. smegmatis* Δ*hup*. If these changes are linked to a role of Hup in gene regulation of enzymes involved in lipid biosynthesis, this might be visualized by microarray comparisons of *M. smegmatis* wild-type and Δ*hup* strains. The *M. smegmatis* genome

has been sequenced and annotated, making this microarray analysis a feasible approach to identifying any genes that may be regulated either directly or indirectly by Hup.

Hup may also be involved in cellular attachment and it is possible that dispersal of mutant cells in liquid lacking detergent could be due to the absence of the Hup protein from the *M. smegmatis* cell wall. It might be possible to mimic the lack of Hup binding in the cell wall by extra-cellular addition of an anti-Hup antibody when wild-type *M. smegmatis* is grown in liquid without Tween 80. The binding of the antibody to the external portion of the cell wall associated Hup could potentially diminish the association of mycobacterial cells. A similar technique was used to demonstrate the involvement of Hup in binding to laminin (7). Along similar lines, an adhesion assay could be performed to determine whether loss of Hup diminishes the ability of *M. smegmatis* to adhere to and be taken up by macrophages or other mammalian cells. If loss of Hup decreases the ability of *M. smegmatis* to attach to potential host cells, this would implicate Hup as a potential virulence factor. If loss of Hup has no affect on the ability of *M. smegmatis* to adhere to mammalian cells this suggests that the *in vitro* studies linking Hup to binding of laminin might not be representative of what occurs in *vivo*. Ultimately the studies described above could shed some light on the potential role of Hup as an adhesion protein.

As Hup might play a part in the formation of the *M. smegmatis* cell wall, an antibiotic sensitivity assay was performed to determine whether alterations in the cell wall affected the sensitivity of *M. smegmatis* Δ*hup* to specific antibiotics. It was shown here that *M. smegmatis* Δ*hup* is more sensitive to the front-line antimycobacterial isoniazid (INH). INH sensitivity is of particular interest to mycobacteriologist since

INH-resistance is a common feature of all multiple-drug resistant (MDR) and extensively drug-resistant (XDR) strains. There are three general hypotheses that could account for this increase in INH sensitivity. First, changes in the cell wall of *M. smegmatis* Δ*hup* might generate an increase in the permeability to INH. This appears unlikely since none of the other antibiotics tested (kanamycin, ethambutol, and rifampicin) demonstrated such a change in sensitivity (data not shown). Also, ethambutol is similar to INH in that it is expected to enter the cell in a similar manner (11). To further demonstrate that no change in cell wall permeability has occurred, a permeability assay could be performed. This assay could measure the uptake of two C^{14}-labeled compounds: a hydrophobic compound chenodeoxycholic acid and a hydrophilic compound glycerol (1). A change in permeability would be detected by changes in the intracellular amounts of one of these compounds relative to the wild-type strain of *M. smegmatis*.

The second hypothesis is that loss of Hup increases the amount of KatG that is produced. As a prodrug, INH must first be activated by the enzyme KatG, and therefore an increase in the amount of KatG could lead to a greater rate of conversion from the INH prodrug to the active form. This hypothesis could be tested by comparing the levels of *katG* mRNA in wild-type and Δ*hup* mutant strains using reverse transcriptase polymerase chain reaction. If more *katG* mRNA is identified in *M. smegmatis* Δ*hu*p this could lead to an increased level of activated INH inside the cell and therefore increased killing. An equal concentration of *katG* mRNA in *M. smegmatis* wild-type and Δ*hup* cells would suggest that alteration of INH activation through increased expression of *katG* is not the reason for the increased sensitivity of *M. smegmatis* Δ*hup*. A final hypothesis is that an increase in sensitivity to INH could be due to the specific action of INH. INH

targets an enzyme involved the synthesis of mycolic acids, which are the primary components of the mycobacterial cell wall. It is possible that loss of Hup leads to changes in the cell wall mycolic acid composition that make the cell more susceptible to inhibition of INH. The previously described TLC *M. smegmatis* experiment could provide additional evidence of what changes are occurring in the cell wall. In addition, performing TLC after wild-type and Δ*hup* strains of has been exposed to bacteriostatic concentrations of isoniazid could provide clues to the differing effect of INH on wild-type *M. smegmatis* compared to *M. smegmatis* Δ*hup*.

In addition to altered INH sensitivity, the *hupB* mutant shows increased sensitivity to UV irradiation. This increased sensitivity could be the result of increased DNA damage occurring in *M. smegmatis* Δ*hup*. One way to test this hypothesis would be the use of an endonuclease sensitive site assay to determine the number of UV-induced pyrimidine dimers in both strains of *M. smegmatis* (6). Other prokaryotic histone like proteins have been linked to an involvement in DNA repair (3, 4). It seems unlikely that in *M. smegmatis* Hup is similarly involved because no increase in sensitivity to the DNA-damaging agents mitomycin C or H_20_2 was detected in *M. smegmatis* Δ*hup* (data not shown). However, if Hup is involved in some aspect of DNA end-joining, this could be detected by evaluating the efficiency of electroporation of linear DNA into both wild-type and Δ*hup* strains of *M. smegmatis*. If *hup* is involved in end-joining, it could be expected that *M. smegmatis* would have a lower transformation efficiency to linear DNA compared to wild-type *M. smegmatis*. Another possible explanation for the increase of *M. smegmatis* Δ*hup* to UV light is that loss of Hup caused changes in the cell wall that increased the penetrance of the UV light into the cells. This would potentially

correspond to an increase in pyrimidine dimers as determined through the endonuclease sensitivity method described above. Determining specifically that this increase in DNA damage is due to changes in the cell wall could be very challenging since it would involve recreating a wild-type cell wall in *M. smegmatis* that is lacking cellular Hup.

Finally since *hup* appears to be an essential gene in *M. tuberculosis,* the lack of a stronger phenotype in *M. smegmatis* Δ*hup* could be due to redundancy of function in *M. smegmatis* (8). If this is the case, then loss of Hup in *M. smegmatis* could lead to an increase in expression of this alternative gene. In *E. coli*, for example, loss of H-NS leads to an increase in the expression of *stpA*, as StpA compensates for the absence of H-NS (2). A thorough examination of a microarray analysis comparing *M. smegmatis* wild-type with *M. smegmatis* Δ*hup* could indicate an increase in the expression of this complementary gene. Examination of genes with increased expression in *M. smegmatis* for those that code for proteins with similar characteristics to Hup, such as a highly basic nature, a high concentration of lysines, alanines and prolines, and a possible DNA binding motif could identify the protein that shares the function of Hup in *M. smegmatis*. If such a gene is identified that shares similar characteristics to Hup, a double knockout could be created to determine the effect of loss of both *hup* and this additional gene. A similar type of redundancy is seen in the Hu proteins of *E. coli*. Loss of one of the members of the Hu heterodimer leads to an increase in the expression of the remaining member (10).

While the initial goal to determine whether *M. bovis* and *M. tuberculosis* differ in the ability to withstand stress was addressed in the work described in this dissertation, ascertaining the exact cause for this difference could prove challenging. The extreme

importance of the thick lipid rich cell wall of mycobacteria in allowing these organisms to survive harsh environments suggests that any differences seen could be the result of differences in cell wall composition. The studies performed on *M. bovis* and *M. tuberculosis* did have the advantage of drawing our attention to the possible importance of the histone-like protein Hup. The initial hypothesis addressed was that in *Mycobacterium*, Hup is involved in a stress response. Although the results presented in this dissertation do not fully elucidate the role for Hup in such a response, they did raise additional questions, such as why does the loss of Hup increase the sensitivity of *M. smegmatis* to UV light and the front-line drug isoniazid. Future work on this project could be directed towards understanding whether the increased sensitivity to UV light leads to an increase in UV damage and whether Hup is involved in DNA end-joining *in vivo*. In addition, future studies could address the reason for the increase in INH sensitivity by looking at whether this is a factor of increased permeability, increased *katG* expression, or due to an increased fragility of the *M. smegmatis* Δ*hup* cell wall. Combining the information learned from the experiments described above with studies examining the role of Hup in cell adhesion and the role of Hup in gene regulation could help pinpoint the exact function and therefore importance of Hup in *Mycobacterium*.

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CHAPTER SIX

Appendices

Protocols

Extraction of Total Cell Wall Lipids

M. smegmatis was grown to stationary phase in 250ml 7H9. Cells were then centrifuged at 3,000 rpm for 10 minutes. Cell pellets were resuspended in 32 μl of chloroform: methanol (2:1) per 50 μg wet cell weight. The cell suspension was heated to 55°C for 1 hour then centrifuged for 10 minutes at 3000 rpm. The supernatant was saved and the pellet was extracted a further 1-hour at 55°C by the addition of 32 μl of chloroform: methanol (2:1) per 50 μg wet cell weight. The suspension was centrifuged again and the supernatant was combined with the previously saved supernatant. This was then placed in a glass beaker and allowed to dry at room temperature. The dried lipids were resuspended in 1 ml of chloroform: methanol (2:1) and analyzed by TLC.

Extraction of Polar Lipids

M. smegmatis was grown to stationary phase in 250ml 7H9. Cells were then centrifuged at 3,000 rpm for 10 minutes. The cell pellets were weighed and 2ml of methanol:0.3% NaCl (100:10) and 1ml of petroleum ether was added to 50 mg of wet weight. The cell suspension was incubated for 15 minutes in a 37°C shaking incubator. The suspension was removed from the incubator and allowed to stand for 10 minutes. The lower layer was separated and heated in a boiling water bath for 5 minutes. Next, 2.3 ml of chloroform-methanol-0.3%NaCl (9:10:3) was added to the mixture and incubated in a 37°C shaker incubator for one hour. To separate the solvent extract from the cell

biomass, the mixture was centrifuged at 3000 rpm from 10 minutes. The supernatant was saved. The biomass was further extracted by the addition of 0.75ml of chloroform-methanol-0.3%NaCl (5:10:4) for 30 minutes. Following centrifugation at 3000 rpm for 10 minutes, the supernatant was retained and mixed with the previous saved extract. The solvent extracts were mixed with 1.3 ml of chloroform and 1.3 ml of 0.3% NaCl for 5 minutes and then centrifuged again at 3000 rpm for 10 minutes. The lower organic layer was evaporated to dryness and resuspended in 300 μl of chloroform: methanol (2:1). TLC was used to analyze the polar lipid suspension.

Extraction of Mycolic Acids

M. smegmatis was grown to stationary phase in 250ml 7H9. Cells were centrifuged at 3000rpm for 10 minutes. The resulting cell pellet was weighed and 2ml of 15% tetrabutylammonium hydroxide was added to 50 mg of wet cell weight. The cell suspension was heated to 100°C for 12 hours. After the suspension was allowed to cool, 2 ml of water were added. This was followed by the addition of 1ml dichloromethane and 250 μl of iodomethane. The mixture was stirred for 30 minutes at room temperature. The upper layer was removed and the lower organic layer was washed with 3 ml of 1M HCl followed by 3 ml of water. The organic layer was then dried and dissolved in 0.5ml of dichloromethane. This crude MAME suspension was transferred to a microcentrifuge tube and dried. The dried residue was dissolved in 0.2ml of toluene and 0.1 ml of acetonitrile. An additional 0.2ml of acetonitrile was then added. This suspension was incubated for 1 hour at 4°C. The precipitated MAMEs were removed by a brief

centrifugation at 4°C and resuspended in 200 μl of dichloromethane. The MAMEs were analyzed using TLC.

Extraction of Apolar Lipids

M. smegmatis was grown to stationary phase in 250ml 7H9. Cells were centrifuged at 3000rpm for 10 minutes. The resulting cell pellet was weighed and resuspended in 2ml of methanol:0.3% NaCl (100:10) and 1ml of petroleum ether per 50 mg of wet weight. The cell suspension was incubated in a 37°C shaker incubator for 15 minutes. The upper layer was removed saved. The lower layer was extracted with an additional 1 ml of petroleum ether for 15 minutes. The upper layer from this second extraction was combined with the saved extract and evaporated to dryness. The resulting apolar lipids were dissolved in 250μl of dichloromethane and analyzed by TLC.

Thin Layer Chromatography

TLC was performed using AL SIL G/UV (Whatman) plates cleaned with acetone and allowed to dry. The plate was then activated at 110 °C for 30 minutes. Plates with spotted with sample approximately 1 cm from the bottom and the run in the appropriate solvent. Apolar lipids were separated using petroleum ether: ethyl acetate (98:2). Polar lipids were separated using chloroform: methanol: water (65:25:4). Mycolic acids were separated six times using petroleum ether: diethyl ether (95:5). Once the solvent reached

2 cm from the top of the plate, the run was stopped and the plates were allowed to dry before development. Visualization of all lipids was performed by submersing the plates in 5% phosphomolibdic acid in ethanol for 10 minutes. The plates were baked at 110° C for 10 minutes.

Isolation of *M. smegmatis* Genomic DNA

M. smegmatis cells were grown in 50 ml of 7H9 to stationary phase. Cells pellets were collected centrifugation at 3000 rpm for 10 minutes. Cell pellets were resuspended in 1ml of Tris-HCl-EDTA then treated with 20 μl of lysozyme (10 ml/ml) for 1 hour at 37°C. Following cell lysis, 12 μl of proteinase K (10 mg/ml) was added and incubated at 55°C for 1 hour. This incubation was followed by the addition of 140 μl 10 % SDS and 200μl 5M NaCl. After mixing thoroughly 160 μl of heated CTAB/NaCl solution was added and the mixture was incubated for another 10 minutes at 55°C. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly. This mixture was then centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous phase was saved and transferred to a fresh microcentrifuge tube. DNA was precipitated by the addition of half the starting volume of ice-cold isopropanol. Following a 30-minute incubation at 4°C the sample was centrifuged and the nucleic acid pellet was washed with 500 μl of ethanol. After the washing, the pellet was allowed to dry and resuspended in 50 μl of TE buffer with 1μl of 4 μg/ml RNase.

Preparation and transfer of gel to nylon membrane for Southern

Digested chromosomal DNA was separated on a 1% agarose gel for 24 hours at 10 volts. The gel was then 100 ml of 0.25 NaCl for 10 minutes and washed twice with water. This was followed by a 15 minute incubation in 100 ml of 0.5M NaOH and 1.0 M NaCl. After washing twice with water, the gel was incubated for 30 minutes in 100 ml of 0.5M Tris (pH=7.4). The prepared gel was then transferred to a nylon membrane.

A glass tray was filled with 4X SSC transfer buffer (20X=17.53% NaCl, 8.82% sodium citrate pH=7.0). A second glass stand was set up inside the original glass tray. A transfer wick of Whatman No. 3 filter paper was placed on the stand so that its ends dipped into the transfer buffer. Two pieces of Whatman filter paper were cut to the size of the gel and placed on top of the wick. The gel was then added to the filter paper and a piece of Nylon membrane was placed on top of the gel. The nylon membrane was previously prepared by soaking in methanol for 1 minute and washed with transfer buffer for 5 minutes. Another two pieces of Whatman paper were placed on top of the membrane. This was then weighed down with a 6-inch stack of paper towels on top of which a heavy book was placed. Transfer took place at room temperature for 12 hours.

Hybridization of Probe to Membrane

Following transfer of DNA, the membrane was cross-linked at 50 mJoule/sec on a Biorad GS Gene Linker UV chamber (setting C2). The membrane was then placed in hybridization buffer (1mM EDTA, 0.5 M $NaPO_4$, 7%SDS) at 60 °C for 4 hours. The

probe was prepared for hybridization by the addition of 50 μl of water to 45 μl of the probe and then boiled for 15 minutes. The boiled probe was added to 22 ml of hybridization buffer and placed with the membrane in the hybridization chamber for 12 hours at 60°C.

Visualization of Southern

After hybridization the membrane was washed twice in low stringency buffer (2X SSC+ 0.1% SDS) for 5 minutes at room temperature. Then washed twice with high stringency buffer (0.1X SSC+ 0.1% SDS) for 15 minutes at 68°C. The membrane was then washed once with phosphate buffered saline for 2 minutes at room temperature. Blocking of the membrane was performed using Roche blocking solution (1 gm blocking reagent+100 ml of PBS) for 30 minutes at room temperature. 4 μl of alkaline phosphatase labeled antibody reactive against the DIG labeled probe was mixed with 20 ml of blocking solution and added to the membrane for 30 minutes at room temperature. Following the addition of the antibody, the membrane was washed twice for 15 minutes with PBS and then placed in detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH=9.5) for 3 minutes. The membrane was developed in 50 ml alkaline phosphate buffer plus 330 μl BCIP and 330 μl NBT for 15 minutes in the dark. The development was stopped by placing the membrane in TE buffer (10mM Tris, 1mM EDTA, pH=8).

Isolation of *M. smegmatis* RNA

M. smegmatis cells were grown to the appropriate optical density in 50 ml of 7H9. Cell pellets were collected at 3000 rpm for 10 minutes and resuspended in 1 ml Trizol (Invitrogen). Trizol suspension was added to glass beads and bead beat 3 times at a speed of 6.5 for 45 seconds. The cell lysate was separated from the cell debris by centrifugation at 14,000 rpm for 10 minutes. The supernatant was removed and mixed with 300 μl chloroform: isoamyl alcohol (24:1). The solution was mixed by periodic inversion for 2 minutes then centrifuged at 4° C for 10 minutes at 14,000 rpm. The aqueous layer was removed and mixed with an equal volume of ice-cold isopropanol. The mixture was incubated at 4°C overnight and then centrifuged at 14,000 rpm for 20 minutes. The pellet was washed with 70% ethanol and then air-dried. RNA was resuspended in 50 μl of RNase-free water.

DNase treatment of RNA samples

The 50 μl RNA suspension was mixed with 0.1M sodium acetate, 5 mM magnesium sulfate, and 50 U of DNase for 3 hours at 37°C. This treatment was performed a total of two times. After each treatment the RNA was cleaned using an RNeasy Mini Kit (Qiagen). A PCR was performed to check for DNA contamination. If a product was generated then another round of DNase treatment was performed.

cDNA synthesis and RT-PCR

A cDNA synthesis reaction was performed by mixing 2 μg of RNA with 5μl of 5 mM dNTPs and 5 μl of 75 ng/μl random hexamer primers. The mixture was incubated at 65°C for 5 minutes then chilled on ice for 5 minutes. The sample was then centrifuged at 14,000 rpm for 1 minute at 4° C. Each sample was then combined with a reverse transcription master mix (8 μl 5X first strand synthesis buffer, 2μl 0.1 M DTRT, 2 μl 40 U/μl Rnase-out, 2 μl Rnase-free water). The sample was then separated into two reactions. To one of the reaction mixes, 1 μl of SuperScripte III reverse transcriptase (Invitrogen) was added. Both samples were incubated at room temperature for 5 minutes and then heated to 50 °C for 1 hour. The samples were then briefly centrifuged and incubated at 70°C for 15 minutes. The samples were then used as template in the appropriate PCR.

MIC Determination

M. smegmatis cells were grown in 7H9 to an OD_{600}=0.2-0.3 then diluted 1000 fold. 50 μl of 7H9 was added to each well in a 96 well round-bottom plate. The first column was left empty. To the first column, 100 μl of the antibiotic at twice the desired initial concentration was added. Then using a multichannel pipettor, 50 μl was transferred to each column starting with column 1 and ending with column 11. The 50 μl removed from column 11 was discarded. Next, 50 μl of the diluted bacterial cells were added to each well (approximately $1*10^4$ cells/well). Following 48 hrs of incubation at 37°C, the plate was read as either growth or no growth using a dissecting scope.

Preparation and transformation of *M. tuberculosis*

M. tuberculosis was grow in 50ml 7H9+ADC to an OD_{600}=0.5. Cell pellets were collected by centrifugation at 3000 rpm for 10 minutes at room temperature. Pellets were resuspended 10% glycerol at half the volume of the original starting culture. After centrifugation cells were resuspended again in 10% glycerol at the same volume as used previously. Cell pellets were collected by centrifugation once more and resuspended in 10% glycerol at one quarter of the starting volume. Following a final centrifugation, cell pellets were resuspended in 1-2 ml of 10% glycerol. At this step a thick paste should be obtained. 50-75 μl aliquots were prepared and stored at -80°C until use.

Transformation of *M. tuberculosis* was conducted at room temperature using 0.2cm cuvettes. 0.1 to 1 μg plasmid DNA in 1-2 μl was added to 50-75 μl of electrocompetent cells and incubated for 5 minutes. The electroporator was set to 2.5 kV, 1000 Ω, and 25 μF. Following electroporation a time constant of at least 12 should be obtained. Immediately after electroporation, 900 μl of 7H9+ADC was added and the mixture was incubated for 24 hours at 37°C prior to plating on the appropriate antibiotic.

Preparation and transformation of *M. smegmatis*

M. smegmatis was grown in 500 ml of 7H9 to an OD_{600}=0.5-1.0. The cultures when then chilled on ice for 15 minutes before centrifugation at 6000 rpm for 10 minutes at 4°C. The resulting cell pellet was resuspended in half the volume of ice cold 10% glycerol and chilled for 10 minutes. Following another round of centrifugation at 6000 rpm for 10 minutes at 4°C, the cell pellet was resuspended in one fourth the volume of ice cold 10% glycerol and chilled for 10 minutes. After another round of centrifugation,

the cell pellet was resuspended in 1-5 ml of ice cold 10% glycerol. Aliquots of 400 µl was prepared and stored at -80°C until use.

Electroporation of *M. smegmatis* was conducted by mixing 4 µl of plasmid DNA (0.1-1 µg) with 400 µl of competent cells. The mixture was then chilled on ice for 5 minutes and then transferred to a 0.4 cm cuvette. Electroporation occurred using the following settings: 2.5 kV, 25 µF and 1000 Ω. Immediately following electroporation 1 ml of 7H9 was added and the culture was incubated at 37°C for 4hrs. Transformants were recovered by plating n 7H11 supplemented with the appropriate antibiotic.

Use of tetracycline inducible anti-sense system to knockdown gene expression in *M. tuberculosis*

INTRODUCTION

Attempts were made to knockdown the expression of *hupB* in *M. tuberculosis* using an anti-sense approach. This technique involves the production of an anti-sense strand of mRNA specific to the gene of interest that would bind to and prevent translation of the sense *hupB* mRNA. Antisense technology has been used for the purpose of knocking down expression of other genes in mycobacteria (1, 2, 5, 8, 11). These approaches have used an antisense construct cloned behind a constitutive heat shock promoter (1, 5), an inducible acetamidase promoter (8), an inducible tetracycline promoter (2), and a native promoter for the antisense gene (11). The approach taken in this project involved the use of a tetracycline inducible promoter. Although this approach was ultimately unsuccessful the work described below details the system that was used and the protocols followed.

MATERIALS AND METHODS

Generation of anti-sense construct. The *hupB* gene was amplified by PCR using primers $pMind_{anti}F$ (**ACTAGT**TCGGTTC CAAACGAGGACCA) and $pMind_{anti}R$ (**GGATCC**CATTGCCACCGTGCAAA GGGTA). The PCR product was then cloned into the SpeI and BamHI sites of pMind (2) to produce plasmid $pHup_{anti}$. The plasmid pMind in is a mycobacterial shuttle plasmid with origins of replication for both *E. coli* and *Mycobacterium*. pMind contains Tetracycline repressor and operator sites upstream

of the multiple cloning site. $pHup_{anti}$ was electroporated into *M. tuberculosis* and plated on 7H11+OADC+Hyg (50 μg/ml).

Inducing anti-sense knockdown. *M. tuberculosis* was grown to mid-log phase (OD_{600}=0.5) in 7H9+ADC+Tween80. Tetracycline was added at concentrations for 10 ng/ml to 4000 ng/ml. Samples were taken at 1 day and 4 days and analyzed by spectrophotometer and SDS-PAGE.

RESULTS AND DISCUSSION

Before electroporation the plasmid construct was sequenced to insure that proper cloning occurred. The sequencing reaction revealed no mutations that might interfere with the production of the anti-sense *hupB* mRNA. Despite variations in starting OD, length of induction, and concentration, no apparent knockdown of hupB was visualized by SDS-PAGE (data not shown). In addition knockdown of hupB was expected to slow growth of the mycobacterial cells, which was not detected by spectrophotometric readings (data not shown). The highest concentration of Tet tested did result in some decrease of OD_{600} in both experimental and control cultures due to killing by the antibiotic (data not shown).

The failure to generate a successful knockdown here is likely attributed to three potential problems: inefficiency of the system used, the concentration of antisense mRNA produced, and the specific antisense mRNA region chosen. First, the system used here

was optimized in *M. smegmatis* and not *M. tuberculosis* and may not work as efficiently in *M. tuberculosis*. Second, successful gene inhibition requires a high enough concentration of antisense mRNA to block translation the sense mRNA. As an abundant *Mycobacterium* protein, representing 7-10% of the total protein (6), the amount sense mRNA may have been significantly greater than the antisense mRNA generated. It may be necessary to increase in the concentration of antisense mRNA to increase the efficiency of the gene inhibition (4, 7). Third it may be necessary to optimize the exact region of antisense mRNA produced in order to get efficient binding to the sense strand (3, 4). Since mRNA is prone to the formation of secondary structure, which could interfere with the formation of duplex RNA (10), it may be useful to generate a library of smaller antisense constructs for binding numerous regions of the sense construct. Instead of using the entire gene to create the antisense mRNA, screening this library of smaller constructs could identify a more effective antisense mRNA. Programs are available for predicting secondary structure that can be useful for selecting the antisense sequence (10). Rasmussen *et al*. recommend that an antisense construct cover the start codon, the Shine- Dalgarnon sequence and at least 5% of the gene (9). Ultimately the lack of success here could be due to a combination of the three potential problems mentioned above.

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