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**IDENTIFICATION AND CHARACTERIZATION OF A
MYCOBACTERIUM TUBERCULOSIS GENE THAT ENHANCES
MYCOBACTERIAL SURVIVAL WITHIN MACROPHAGES**

**By
Jun Wei**

**A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY**

**In Partial Fulfillment of the Requirements
For the Degree of**

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2001

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entitled Identification and Characterization of a Mycobacterium
tuberculosis Gene that Enhances Mycobacterial survival
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ACKNOWLEDGEMENTS

I would like to acknowledge my advisor, Dr. Richard Friedman, for his patience and excellent guidance during my graduate career in his laboratory. I feel a great pleasure in expressing my thanks to him for teaching me the process of scientific thought, for his unwavering support and encouragement of me throughout the course of this study.

Special thanks go out to my honorary advisor, Dr. James Moulder, for his timely and invaluable advice to this work. It has been a pleasure and an honor to work with him.

Additionally, I would like to thank my committee, Dr. Junetso Ito, Dr. Lynn Joens, Dr. Leland Pierson III and Dr. Glenn Songer, for their suggestions, assistance with my dissertation project.

I would like to extend my thanks to members of Friedman lab, including Dr. John Dahl, Esteban Roberts, and Amy Carlson, for their help with experiments.

DEDICATION

I would like to dedicate this dissertation to my wife, Hong Sun, who has not only given me her unconditional love, friendship and encouragement, but has also helped me to prepare my work. This dissertation is also dedicated to my parents, Wei Wenxuan and Wang Xuemei. Thank them for giving me with such incredible amount of support and encouragement while I pursued my educational goals. It is their guidance and inspiration that has allowed me to accomplish my dreams.

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ABSTRACT

The virulence of *Mycobacterium tuberculosis* (Mtb) depends on its ability to multiply and survive within host macrophages. In screening for Mtb genes that play a role in the intracellular survival, a Mtb gene (*eis*) was identified that enhanced survival of *Mycobacterium smegmatis* in both human monocytes and in the human macrophage-like cell line U-937 when introduced on the multi-copy plasmid pOLYG. When a single chromosomal copy of *eis* was introduced into *M. smegmatis*, using an integrative vector, the construct still exhibited increased intracellular survival in U-937 cells. The *eis* gene was found in the genomic DNA of various *M. tuberculosis* strains and of *Mycobacterium bovis* BCG but not in that of *M. smegmatis* or 10 other mycobacterial species. Western blot analysis showed that the *eis* gene could produce a 42-kD protein product in both *M. smegmatis* and *M. tuberculosis*. The role of the *eis* gene in *M. tuberculosis* intracellular survival and multiplication was investigated by inactivation of the *eis* gene via allelic exchange. A mutated *eis* allele (*eis::hyg*) was delivered at the *eis* locus, using the suicide vector pMJ10, in both Mtb strains H37Rv and H37Ra. Complemented mutants were also constructed by reintroducing a wild-type *eis* into the chromosome *attB* site. Southern and Western blot analysis demonstrated that the *eis* gene was disrupted and no Eis protein was produced, and that complemented strains regained the ability to produce Eis. Wild-type *M. tuberculosis*, *eis* knockout mutant and complemented strain were then evaluated for their capacity to survive and multiply within U-937 cells. The *eis* mutant survived and multiplied, as its parental strain, in U-

937 cells over a 7-day period. These findings suggest that *eis* may not be required for the short-term multiplication of *M. tuberculosis* in U-937 cells. Further *in vivo* study needs to be done to clarify the role of *eis* in the pathogenesis of tuberculosis.

INTRODUCTION

SCOPE OF THE PROBLEM

Tuberculosis (TB) is one of the oldest infectious diseases. It has contributed significantly to the morbidity and mortality of populations throughout the world from ancient times through to the nineteenth century (34). As economic conditions and public health improved in many nations of the world during the twentieth century, the incidence of tuberculosis began to decline (89). In 1882, Robert Koch isolated *Mycobacterium tuberculosis* and demonstrated that it was the etiological agent of tuberculosis (169). In the early 1920s, Calmette and Guérin isolated an attenuated culture of *Mycobacterium bovis*, a member of the TB complex, named bacillus Calmette-Guérin (BCG) (46). This BCG vaccine has been widely used since then, and it is presently the only available vaccine against TB. The extensive vaccination with BCG was believed to be the key to solve the TB problem in the third world. BCG vaccine generally induces high levels of acquired resistance in animal models of TB (278). The efficacy in human populations, however, remains a matter of controversy. Some trials have demonstrated efficacies as high as 80% whereas others have demonstrated very low levels of protection (64). Identification of effective chemotherapeutic agents in the 1940s and 1950s further accelerated the decline in incidence of the disease, although these declines were most noticeable in populations of developed nations. However, despite these early advances, the reductions in the prevalence and incidence of tuberculosis have not occurred throughout the twentieth

century. In most developing countries, the incidence rate remains high (105). Among the developed nations, the incidence of tuberculosis began to increase in the mid-80s (49). In fact, tuberculosis is now the leading cause of death due to a single infectious agent among adults throughout the world (170). TB infects one-third of the world's population and kills more than 3 million people each year (34, 324). This ever-growing death toll prompted the World Health Organization (WHO) to declare tuberculosis a global health emergency in April 1993 (313). Indeed, at current rates, it has been estimates that up to one-half billion people will suffer from tuberculosis in the next 50 years and the infection will prove fatal to more than 20 million of them (314).

Several reasons have been suggested for the discrepancy between the early successes in containing tuberculosis and the present alarming situation. These include the difficulty in diagnosing the disease at early stages of infection, the lack of patient compliance in taking antibiotics for the duration of the treatment regimen, and the low efficiency of the BCG vaccine against pulmonary tuberculosis. Moreover, the emergence of the worldwide epidemic of AIDS has contributed significantly to the increase in tuberculosis incidence due to accelerated progression of the disease and higher mortality rates (19, 35). The increasing number of new cases of tuberculosis in some developed countries is most probably a side-effect of the AIDS epidemic. Finally, the appearance of multi-drug resistant strains of *M. tuberculosis* is also contributing to the worsening impact of this disease. Cases of tuberculosis are often increasingly difficult to treat because of the appearance of TB strains (as many as 15% of all tested

isolates for example in Latvia) that are resistant to all front-line anti-tuberculous drugs (312).

BACTERIOLOGY OF TUBERCULOSIS

M. tuberculosis belongs to the genus *Mycobacterium* (29). The genus *Mycobacterium* is divided into two major groups: the slow growers and the rapid growers. The best known property of the genus is the ability of the bacterial cell to resist decolorization by weak acids after staining, hence the term "acid-fast bacilli". Currently, over 60 species of mycobacteria have been well defined. Most species of mycobacteria are free-living environmental organisms that very rarely or never cause disease. A relatively small number of slow-growing species are obligate pathogens. The most important of these are members of the *M. tuberculosis* complex which is comprised of four species: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (18). These species can be differentiated on the basis of their host range, virulence for man, and physiological/biochemical characteristics. All four species are very similar genetically and they invade and survive in macrophages in similar ways. On the basis of DNA-DNA hybridization studies, these species could be considered simple varieties of *M. tuberculosis* (129). Genomic DNA analysis suggests a degree of relatedness to the last common ancestor of *M. tuberculosis* as follows: last common ancestor > *M. bovis* > *M. bovis* BCG > *M. africanum* > *M. microti* > *M. tuberculosis* (136, 282).

M. tuberculosis is a facultative intracellular pathogen, preferentially infecting and multiplying within human mononuclear phagocytes. But it can grow both intra- and

extracellularly (18). It causes most cases of human tuberculosis. *M. africanum* causes a proportion of the human tuberculosis, particularly in parts of Africa. *M. bovis* primarily causes tuberculosis in cattle and many other species of animals. In humans, it causes a disease that is clinically indistinguishable from that caused by *M. tuberculosis*. *M. microti* is responsible for naturally acquired tuberculosis in the vole and is never encountered in human clinical cases (210).

M. tuberculosis is a thin rod with rounded extremities, 2-5 μm long and 0.2-0.3 μm thick, nonmotile, without capsule or spore. It is difficult to stain via the usual methods, although it belongs to the Gram-positive bacteria. But stained with the reference carbol fuchsin or Ziehl-Neelsen method, it resists decolorization with strong mineral acids and alcohol, hence is an acid-fast bacillus. It appears under microscopic examination as a slightly curved or straight, small red or pink rod (210).

The mycobacterial cell wall is composed of peptidoglycan, covalently linked via phosphodiester bonds to arabinogalactan which is esterified at its distal end with fatty acids containing 60 to 90 carbon atoms, named mycolic acids. Mycolic acids play a major role in the acid-fastness of mycobacteria (21). Another group of important cell wall components is the acylated trehalose-dimycolate (34). They may be important for virulence, since most virulent strains of *M. tuberculosis* elaborate strongly acidic sulfolipids, which may be involved in inactivating the macrophage phagosome. In addition to the above components, lipoarabinomannan (LAM), which is anchored in the mycobacterial cell membrane, is thought to extend all the way to the bacterial surface. The arabinose termini of LAM from *M. tuberculosis* and *M. bovis* are capped with a

few additional mannose residues (manLAM) in contrast to LAM from fast-growing nonpathogenic mycobacteria (araLAM) (51). This difference in capping significantly affects production of cytokines by macrophage (52, 255, 256).

Because of its thickness and high lipid content, the mycobacterial cell wall is much more impermeable to hydrophilic molecules than other bacteria. The high lipid content of the cell wall is also responsible for the resistance of mycobacteria to decontamination procedures with acids, sodium hydroxide, and/or detergents. However, *M. tuberculosis* is as susceptible as other bacteria to heat, x-rays, UV rays, and 70 % alcohol. *M. tuberculosis* remains viable for weeks at 4 °C and for years at -70 °C (34).

M. tuberculosis is a slow-grower, the generation time of the microbes under the best conditions of culture being 13 to 20 h on solid or in liquid medium. It produces colonies after incubation for 3-6 weeks at 35-37 °C. The typical colonies are well developed with a rough surface and a characteristic buff color (210).

M. tuberculosis is a strict aerobe equipped with catalase, peroxidase, and superoxide-dismutase. The growth rate of *M. tuberculosis* is highly dependent upon the oxygen concentration. When oxygen levels are high, as in the tuberculous cavity of the lung, *M. tuberculosis* multiplies rapidly. When oxygen levels are low, as in the caseous foci of the lung in the latent infection, *M. tuberculosis* multiplies slowly or not at all, and this is referred to as “dormant” or “latent” state (139). The persistent state of *M. tuberculosis* under laboratory conditions has been identified. However the evidence linking physiological states of *M. tuberculosis in vitro* with latent human infections still remains circumstantial (132, 139). Previous studies have suggested that the ability to

tolerate anaerobic conditions may be pivotal to the pathogenicity of *M. tuberculosis*. *M. tuberculosis* H37Rv has a lower respiratory rate than the avirulent strain *M. tuberculosis* H37Ra, and the rate of respiration is less inhibited by lower oxygen tension in H37Rv than in H37Ra (153). A lower respiratory rate may provide the bacilli with sufficient time to adapt to lower oxygen tension in preparation for entering a state of dormancy, and ordered metabolic shutdown (309).

Different geographic varieties of *M. tuberculosis* have been observed. In Europe and the United States the organisms grow slowly in culture into well-developed, rough colonies with a characteristic buff color. In Southeast Asia, colonies of *M. tuberculosis* are small and smooth (210). It has been known for some time that strains of *M. tuberculosis* from different parts of the world show considerable variation in their virulence for guinea pigs. For instance, *M. tuberculosis* clinical strains isolated from Southern India are less virulent in a guinea pig model than strains isolated in United Kingdom, with intermediate degrees of virulence elsewhere (203). Whether *M. tuberculosis* varies in its virulence for humans remains uncertain.

PATHOGENESIS OF TUBERCULOSIS

Initial infection with *M. tuberculosis*

TB infection can be initiated by ingestion, inoculation, or inhalation of virulent mycobacteria, inhalation of droplet nuclei containing tubercle bacilli is by far the most common natural route of tuberculosis infection (189). In most cases virulent mycobacteria must first reach the alveolar surface to begin infection through inhalation

of droplet nuclei that are small enough ($\sim 1\text{-}2\ \mu\text{m}$) to reach the lower respiratory tract (252). A single droplet nucleus containing no more than one to three virulent organisms is sufficient to cause infection (189).

Once organisms have made their way into the lung, they have four potential fates (79). The initial host response can be completely effective and kill all bacilli, such that the patient has no chance of developing tuberculosis at any time in the future. Alternatively, the organisms can begin to multiply and grow immediately after infection, causing clinical disease known as primary tuberculosis. Tubercle bacilli may also become dormant and never cause disease at all, such that the patient has what is referred to as a “latent infection” manifested only by a positive tuberculin skin test. Finally the latent organisms can eventually begin to grow, with resultant clinical disease, known as reactivation disease. In otherwise healthy hosts with a latent infection, a study indicated that there is a 5 to 10% chance over a life-time of developing active disease (1).

Binding and phagocytosis of *M. tuberculosis* into macrophages

Resident alveolar macrophages derived from blood monocytes are the first line of defense against pulmonary tuberculosis (270). These phagocytic cells scavenge the alveolar surface and ingest inhaled organisms and particles, and they are capable of inhibiting growth of the bacillus through phagocytosis. However, the innate bacteriostatic and bacteriocidal activities of alveolar macrophages vary with the

macrophage's state of activation, which in turn is influenced by the genetics of the host and by multiple factors in the macrophage's environment (268, 270).

A number of ligand–receptor interactions mediate the entry of mycobacteria into the macrophage. The principal receptors that mediate phagocytosis of tubercle bacilli by human monocytes and macrophages are complement receptors (CR1, CR3 and CR4), mannose receptor, surfactant protein receptors, CD14, scavenger receptor (334) and Fcγ receptors (100). Complement receptors are believed to play a significant role in the efficient uptake of the bacillus by host macrophages (269, 271). Complement receptors interact with C3 deposited on *M. tuberculosis* when the bacterial surface glycolipid trehalose dimycolate (cord factor) activates the alternative complement pathway (249). Pathogenic mycobacteria can also use another recently described unconventional pathway to recruit C2a directly to form a C3 convertase, resulting in C3b opsonization (273). Mannose receptors interact with terminal mannosyl units on the major bacterial surface lipoglycan, LAM (272). It has been shown that LAM can also bind to the endotoxin receptor, CD14, and that sulfatides from *M. tuberculosis* can bind to macrophage scavenger receptors (240). Accumulating evidence shows that host molecules such as human surfactant protein-A (SP-A), which regulates the level of lung surfactant, also mediates mycobacterial binding and enhances phagocytosis by alveolar macrophages (134). In contrast, the contribution of Fcγ receptor-mediated uptake remains uncertain. Cooperation between distinct types of receptors may be required for optimal binding and ingestion of tubercle bacilli. Which receptors are used for phagocytosis of *M. tuberculosis* may also be influenced by the state of differentiation

and activation of the macrophage. But ultimately individual entry pathways may not have a major influence on intracellular survival and growth of mycobacteria (100).

Bacillary growth and early tuberculous lesion

Once phagocytosed, tubercle bacilli may either be destroyed or start to multiply after a lag period of a few days. If phagocytosis is followed by bacterial killing, then the infection is eliminated. However, when the innate microbicidal capacity of alveolar macrophages fails to destroy the initial few *M. tuberculosis* of the droplet nucleus, the tubercle bacilli replicate within the macrophage and cause the cell to rupture. Released bacilli are then taken up by other macrophages in the vicinity. Pro-inflammatory cytokines (interleukin-1, IL-1; IL-6; tumor necrosis factor- α , TNF- α) and chemokines (e.g. macrophage inflammatory protein 1, MCP-1; interferon-inducible protein 10) secreted from the infected macrophage lead to the development of the inflammatory process with the recruitment of monocytes and lymphocytes from the blood stream (82, 208, 251, 294, 295). At this stage, the host is unaffected by the infection and the macrophages/monocytes have not been activated by cytokines and appear incapable of killing virulent *M. tuberculosis* or inhibiting their growth. Thus, the bacillary multiplication cycle is repeated within immature phagocytes. Successive waves of intracellular multiplication followed by lysis of the infected macrophages lead to the formation and enlargement of the primary lung lesion. In the mouse model, the first signs of substantial specific immunity have been reported to emerge after about 2

weeks of infection, and involve the triggering of cytokine release from specific T lymphocytes (80).

During the early stage of uncontrolled growth, some mycobacteria are transported to draining lymph nodes where the pathological process is repeated. The initial lesion and its inflamed draining lymph nodes form the so-called primary complex of tuberculosis. Bacilli are also widely dispersed to distant metastatic sites via the bloodstream (80).

The outcome of disease is determined by a dynamic balance of host and parasite factors (36). In the resistant individual a high level of immunologic activity may be reached rapidly and the disease is consequently controlled in an asymptomatic stage. In the susceptible host, by contrast, the process of bacterial multiplication and cellular recruitment continues, the primary lesion enlarges and some bacteria are transported to the regional lymph nodes, giving rise to a granulomatous reaction.

Infection control

After about 3 weeks, the growth of tubercle bacilli rather suddenly ceases in both sensitive and resistant hosts. This coincides with the development of acquired immune resistance and the formation of characteristic tuberculosis granuloma (223).

Two distinct immune responses mediate acquired immunity to *M. tuberculosis*: cell-mediated immunity (CMI) and delayed type hypersensitivity (DTH) (79). CMI results in the accumulation of large numbers of activated microbicidal macrophages around solid caseous tuberculous foci. Intracellular tubercle bacilli are killed by

cytokine-activated macrophages. These macrophage-activating cytokines include IL-1, IL-2, IL-6, IL-10, IL-12, TNF- α , IFN- γ , and MCP-1 (226). The cytokines activate the bacteriostatic activity of macrophages and accelerate lymphocyte recruitment. As the immune response progresses, monocytes mature into epithelioid cells and multinucleated giant cells surrounded by T-lymphocytes, thereby forming a granuloma (4, 165, 224).

DTH is characterized at the system level by tuberculin skin test reactivity and at the local level by T-cell-mediated killing of *M. tuberculosis*-infected macrophages (81). The DTH response produces a more tissue-damaging response and benefits the host by destroying immature macrophages that provide a permissive environment for bacillary growth. The released tubercle bacilli can survive extracellularly for many years. They can also be ingested and killed intracellularly by T-cell activated macrophages in the vicinity.

As the disease continues, the amplified immune reaction leads to intense inflammation, tissue destruction, caseous necrosis, and formation of a cavitory lesion. At this stage, lysis of macrophages may result in the release of viable mycobacteria into the blood with the generation of metastatic foci in various organs. If the host controls the infection, the lesions are capsulated, sterilized and left as calcified scars. The mycobacteria may also be held in stasis, and survive for many years extracellularly, but they are unable to multiply in the solid caseous necrotic material that forms the center of the developing granuloma (34).

In most infected persons, the immune response maintains the bacillary population in “reactivable sites” in a steady-state dormant level (231). The continuous release of small amounts of mycobacterial antigens from caseous granulomas presumably maintains both tuberculin reactivity and protective immunity. When events such as stress, treatment with steroids or chemotherapeutic drugs, HIV infection, alcohol, or malnutrition perturbs this equilibrium, liquefaction of the caseous center of the granuloma permits extracellular growth and multiplication of *M. tuberculosis*. Several cytokines and other host factors including IL-1, IL-3, IL-6, TGF- β , and prostaglandin E₂, can “deactivate” macrophages and thereby promote mycobacterial growth (306). Presumably, host defenses are inoperative within liquefied necrotic tissue, but the cavitary fluid permits extracellular multiplication of mycobacteria, with large numbers accumulating in tuberculous cavities. Given the baseline mutation rate in mycobacteria, this favors the emergence of drug-resistant tubercle bacilli.

IMMUNITY TO TUBERCULOSIS

Innate immunity to tuberculosis

Macrophages

Macrophages perform various functions in mycobacterial infection (107). They exert mycobactericidal effects via a variety of mechanisms including phagosome-lysosome fusion, and generation of reactive oxygen and nitrogen intermediates to prevent bacillary growth (detailed in next section). Macrophages also process and present mycobacterial antigens to both CD4⁺ and CD8⁺ T cells, which are central to

acquired resistance to *M. tuberculosis*. Macrophages, upon exposure to mycobacteria, produce a large number of cytokines such as interleukin (IL)-1, IL-6, IL-10, IL-12, IL-15, IL-18, TNF- α , and TGF- β (115). Secretion of cytokines with immunoregulatory properties extends the macrophage's role beyond that of antigen-presenting cell and inhibitor of mycobacterial growth (118, 223). The local production of INF- γ and TNF by leukocytes is critical for the differentiation and activation of the recruited monocytes. While activated alveolar macrophages may kill *M. tuberculosis* effectively, immature monocytes recruited from the periphery are thought to be less effective and serve as the tubercle bacilli's preferred host.

Natural killer (NK) cells

NK cells make up 10 to 15 % of the blood mononuclear cell population. They proliferate in response to both monocyte- and T cell-derived cytokines, and are generally involved as a first line of defense against infection. The major importance of this subset may be as a source of INF- γ during early infection (267). It has been demonstrated that TNF produced by the infected macrophage, together with IL-12, are responsible for the triggering of a pronounced production of INF- γ by NK cells (14). NK cells also exhibit non-MHC restricted cytotoxic activity and serve as a major force in the nonspecific host resistance that occurs early during infection before specific immunity develops.

Neutrophils

The neutrophil is a professional phagocyte with a crucial role in the host defenses against infection by extracellular parasites. In the past few years, evidence has shown that neutrophils also play a protective role in the host response to infection by different intracellular parasites such as *Listeria monocytogenes* (6), *Salmonella typhimurium* (69), *Yersinia enterocolitica* (69), *Chlamydia trachomatis* (23), and *Toxoplasma gondii* (265). Recent data from mouse models suggest that neutrophils also play a protective role in mycobacterial infection. Neutrophils are persistently recruited to the sites of mycobacterial infection (276). Further support for a protective role of neutrophils in mycobacterial infections has been provided by the *in vivo* depletion of these cells by monoclonal antibody treatment. In intravenous (i.v.) models of *M. tuberculosis* infection, mice depleted of neutrophils by using antineutrophil monoclonal antibody exhibited increased susceptibility to bacterial growth (5, 236, 241). These results suggest that neutrophils play an indirect, nonphagocytic role during the early period of mycobacterial infection, probably via an effect on innate production of IFN- γ (236).

Cell-mediated immunity to tuberculosis

Natural immune mechanisms – macrophages, natural killer cells, and neutrophils – likely have an important role in the host primary response to *M. tuberculosis*. However, successful elimination of the intracellular pathogen from the host depends mainly on the efficient interaction between infected macrophages and antigen-specific T-cells (98). The crucial contribution of T-cells is underlined by the observation that patients

with impaired T cell function (e.g. patients receiving immunosuppressive therapy, AIDS patients, or the elderly) are at increased risk of developing clinically manifest tuberculosis (99, 222).

The traditional model for the role of T cells in tuberculosis is one in which macrophages present antigens obtained from phagocytosed bacilli to T cells (37). Antigen-activated T cells then secrete cytokines such as INF- γ , which in turn stimulate macrophages to become more effective in controlling mycobacterial growth (225). Recent observations in basic immunology and in studies of the human immune response to mycobacteria require a revision of this simple model. First, besides the central role of CD4⁺ T cells, other T-cell subsets such as $\gamma\delta$ T cells and CD8⁺ T cells are activated by mycobacteria and have complementary roles to those of CD4⁺ T cells (122). Second, T cells can also serve as cytotoxic effector cells against *M. tuberculosis*-infected macrophages. CD8⁺ T cells can also be cytotoxic to target cells and produce cytokines (302).

CD4⁺ T cells

CD4⁺ T cells express the α/β T cell receptor, and they are involved in recognition of antigens that have been processed in phagosomes and presented as small peptide fragments in the context of MHC class II molecules on the surface of antigen-presenting cells such as monocytes, macrophages, or dendritic cells (83). Soluble protein antigens, such as those found in purified protein derivative (PPD), are strong stimuli for CD4⁺ T cells. However, no major antigen recognized by CD4⁺ T cells has

been identified to date that is specific for infection with *M. tuberculosis*. CD4⁺ T cells exert their influence in the immune response to *M. tuberculosis* through secretion of cytokines and cytotoxicity for *M. tuberculosis*-infected macrophages (277).

Through cytokines, CD4⁺ T cells enhance the effector function of macrophages and regulate the IL-2-mediated expansion of $\gamma\delta$ and CD8⁺ T cells. CD4⁺ cells can be divided into two subsets, Th1 and Th2, on the basis of the cytokines they produce. The Th1 cells are important players in the control of tuberculosis infection due to their production of IFN- γ and TNF- α , both of which activate macrophages, as well as IL-2, which causes T cell proliferation. IFN- γ also down-regulates the Th2 response. The Th2 subset produces IL-4, IL-5, and IL-10, which promote antibody production by B cells, and down-regulates the Th1 response (262). Although Th2 responses can be detected, mycobacterial diseases are generally characterized by strong Th1 responses and high levels of IFN- γ (143). IL-12 is the major cytokine that specifically expands the Th1 population and up-regulates its function. The main source of IL-12 during infection is the macrophages which are stimulated by phagocytic events, and by the presence of IFN- γ and TNF- α in the local environment (131). Therefore IL-12 plays an important role in producing the CMI and DTH responses that control the pathogenesis of tuberculosis. The balance between Th1 and Th2 cells is decisive for the outcome of mycobacterial disease (39).

Recent studies have demonstrated the cytolytic activity of CD4⁺ T cells directly against *M. tuberculosis*. It was shown that CD4⁺ T cells clones stimulated with either purified protein derivative (PPD) (145) or live mycobacteria (211) efficiently lyse a

variety of target cells using both the Fas-FasL pathway and the granule exocytosis pathway of cytotoxicity (183). The presence of CD4⁺ cytolytic T cells in bronchoalveolar lavage fluids indicates that they might participate in the local lung immune response to *M. tuberculosis*. Lysis of target cells was restricted by MHC II molecules and interestingly blood monocytes were lysed more efficiently than alveolar macrophages (291). It remains speculative whether the relative resistance of infected alveolar macrophages to lysis offers the bacteria a safe haven in which to evade host defense or is beneficial to the host by inhibiting the spread of released bacteria throughout the lung.

CD8⁺ T cells

Until recently, the role of CD8⁺ T cells in the human immune response to *M. tuberculosis* was not acknowledged (176, 188, 197). CD8⁺ T cells recognize antigens that have been processed in the cytosol and that are presented in the context of MHC class I molecules on the cell surface (147). Other CD8⁺ T cells also recognize nonpeptide antigens (such as mycobacterial lipid antigens) with CD1 molecules (245). Both subsets of CD8⁺ T cells can be cytotoxic to target cells and produce cytokines, although their relative contribution to protection against *M. tuberculosis* is unknown (280).

Cytotoxic activity by CD8⁺ T cells could contribute to protection in at least two ways. Lysis of macrophages incapable of being activated to kill intracellular *M. tuberculosis* could release mycobacteria to be taken up by activated macrophages (121).

CD8⁺ T cells may also directly kill intracellular bacteria and contribute to protection in this way. Experimental evidence has demonstrated that CD8⁺ T cells lyse *M. tuberculosis*-infected macrophages via a perforin-dependent pathway (284). Perforin forms pores in the plasma membrane of target cells, mediates macrophage lysis, and granulysin-dependent killing of *M. tuberculosis* (285).

CD8⁺ T cells also exert protection through another mechanism, presumably cytokine (IFN- γ) production. Studies have shown that all CD8⁺ T cell subsets are potent producers of IFN- γ (291, 293). Because activation by IFN- γ is crucial for the macrophage to eradicate intracellular pathogens, CD8⁺ T cells may contribute to protection by complementing CD4⁺ T cells as a source of IFN- γ (178).

Double-negative (DN) CD1-restricted T cells

Double-negative (DN) (CD4⁻, CD8⁻) T cells make up less than 1% of circulating T cells and can use the nonpolymorphic MHC-like molecule CD1 as the antigen-presenting molecule (101, 246). Recent studies have revealed the effector functions of DN CD1-restricted T cells that contribute significantly to the host response to infection. This includes the release of IFN- γ and expression of cytolytic activity upon contact with target cells pulsed with mycobacterial lipids (27) and glycolipids by CD1 (275). The cytotoxicity of DN CD1-restricted T cells is mediated by Fas-FasL interaction and has no effect on the viability of the mycobacteria (284).

Gamma delta ($\gamma\delta$) T cells

$\gamma\delta$ T cell receptor (TCR)-expressing T cells form a distinct subset of T lymphocytes, comprising 5% of T cells in lymphoid organ and 1 to 5% of circulating blood T lymphocytes. They express CD3, but are CD4-negative, and fewer than 5% express low levels of CD8 (292). There is strong evidence both in humans and in animal models that $\gamma\delta$ T cells participate in the immune response against mycobacteria (90, 163). Functionally, $\gamma\delta$ T cells are very similar to CD4⁺ T cells. They secrete as much IFN- γ , and are equally cytotoxic for macrophages as CD4⁺ cells (11, 40, 302). Studies suggest that $\gamma\delta$ T cells migrate into mycobacterial lesions to participate in the early containment of the infection (20). As disease becomes chronic, Fas-FasL mediated apoptosis clears the majority of *M. tuberculosis*-reactive $\gamma\delta$ T cells (184).

Essential cytokines

Cytokines are key mediator molecules in the expression of acquired immunity in the lungs. Human mononuclear cells, alveolar macrophages, and T cells produce large quantities of cytokines (such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF- α , and TGF- β) in response to *M. tuberculosis* (112). They have the potential to exert potent immunoregulatory effects and to mediate many of the clinical manifestations of tuberculosis (226).

Great attention has been focused on the role of the cytokine IFN- γ , in terms of its ability to activate macrophages to inhibit mycobacterial growth (109, 120). A major role for IFN- γ in mycobacterial host defense has been suggested by a variety of *in vitro*

and animal experiments (70, 114). IFN- γ acts primarily as a macrophage activator. INF- γ mediates its protective effect predominantly by the induction of reactive oxygen intermediates (ROIs) in activated macrophages which are necessary for the killing of the intracellular mycobacteria. IFN- γ might also improve or augment antigen presentation, leading to recruitment of CD4⁺ T cells and/or cytotoxic T cells, which participate in mycobacterial killing (216).

Activated macrophages are a rich source of TNF, and there is good evidence that TNF contributes to their antimicrobial action. The local release of TNF at the site of disease contributes to granuloma formation, control of infection, and eventual mycobacterial elimination (111). In addition, TNF drives the DTH response by inducing the chemokine response that in turn recruits blood-borne monocytes into the lesion (122). If the DTH response is unchecked, it can cause both unnecessary cellular accumulation and tissue damage (226).

IL-1 is produced upon stimulation of human monocytes with *M. tuberculosis*. IL-1 may enhance the early inflammatory responses by inducing macrophages to produce IL-6 and TNF- α and by stimulating T cell proliferation through up-regulation of T cell expression of IL-2 receptors and IL-2 production (130, 243).

Transforming growth factor β (TGF- β) is widely distributed and produced mainly by monocytes and macrophages. Although it has some inflammatory effects, such as enhancement of monocyte chemotaxis and augmented expression of Fc receptors, TGF- β also has important anti-inflammatory effects (76). These include deactivation of macrophage production of ROIs and reactive nitrogen intermediates (RNIs) (308),

inhibition of T cell proliferation (155), interference with natural killer and cytotoxic T lymphocyte function (154), and down-regulation of IFN- γ , TNF- α , and IL-1 release (38). TGF- β has an inhibitory effect on T cell responses to *M. tuberculosis* by inhibiting IL-2 dependent T cell proliferation and IL-2 receptor expression (156, 299).

IL-6 is a potent B cell growth and differentiation factor that induces immunoglobulin production by activated B cells. Limited experimental evidence suggests that IL-6 does not enhance mycobacterial clearance (220). IL-6 antagonizes the antimycobacterial activity of TNF in macrophages infected with *M. tuberculosis* (30).

Human monocytes and alveolar macrophages produce IL-8 upon exposure to live *M. tuberculosis* or mycobacterial LAM (50). IL-8 production is dependent on the production of TNF- α and IL-1 (127). IL-8 predominantly attracts neutrophils, which are a prominent component of the inflammatory infiltrate in the lungs of some tuberculosis patients (329). These findings suggest that IL-8 may contribute to the initial neutrophilic inflammatory response in human tuberculosis.

IL-10 has a critical role in the early anti-inflammatory response against tuberculosis. In human infection, IL-10 appears to be macrophage-derived and its secretion is a specific response to phagocytosis of *M. tuberculosis* (38, 162). IL-10 inhibits proliferation of T cells and IL-2 production by activated T cells, it also downregulates the secretion of proinflammatory cytokines by LAM-activated monocytes (127).

IL-12 is one of the proinflammatory cytokines (as are IL-2 and IFN- γ) produced by macrophages. IL-12 has been shown to be critical in the commitment of naive CD4⁺ T cells to a Th1-type profile of cytokine production (71). This cytokine also acts to enhance the cytolytic activity of CD8⁺ T cells, NK cells, and to augment their production of IFN- γ (72).

Humoral immunity to tuberculosis

It is important to recognize that the pathogenesis of tuberculosis is almost exclusively determined by host T-lymphocyte-mediated cellular immune responses. At the same time, one must acknowledge that antibody-mediated humoral responses to mycobacterial antigens occur in patients with tuberculosis (158). Levels of immunoglobulin-G (IgG) antibody detected by enzyme-linked immunosorbent assays (ELISA) or other immunoassays are usually an indicator of active tuberculous disease (77, 202). Asymptomatic primary infection and minimal pulmonary disease usually are not sufficient to induce a significant antibody response (78). During the course of treatment, antibody levels rise somewhat for the first 1 or 2 months, falling thereafter but remaining detectable for up to several years.

It has been commonly assumed that humoral immunity has little or no role against intracellular *M. tuberculosis*. In recent years, considerable evidence has shown that antibody can mediate protection against many intracellular pathogens (48). Antigen-antibody reactions may play a role in the pathogenesis of tuberculosis in three possible ways (106). First, the antigen-antibody reactions that activate the complement cascade

may be the first antigen-specific host immune response to tubercle bacilli, because this reaction occurs quickly at the local site, in contrast to the antigen-specific T cell response, which occurs more slowly. C5a, released by the complement cascade, is a major chemoattractant for the phagocytes that defend the host against the bacillus. Second, antibodies may add specificity to NK cells by means of the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction (31). In this case, the NK cells would recognize and kill bacilli-laden macrophages, thereby stopping bacillary multiplication within these macrophages. Third, antigen-antibody reactions may contribute to liquefaction and cavity formation, because the phagocytes (locally accumulating due to the production of C5a) contain a battery of digestive enzymes that may liquefy solid caseous material. All of these possibilities, however, remain to be proven.

THE INTRACELLULAR FATE OF *M. TUBERCULOSIS*

Antimycobacterial activities of macrophages

Mononuclear phagocytes have a large repertoire of mechanisms that kill intracellular organisms. It is likely that different combinations of these antibacterial effector mechanisms act in concert to control a given pathogen. However, the mechanisms by which macrophages destroy tubercle bacillus remain unknown (218). It is most likely that ingested *M. tuberculosis* is killed within activated macrophage phagolysosomes. Toxic constituents found within this acidic vesicle include

lysosomal hydrolases, ROIs, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and RNIs, such as nitric oxide (NO) and nitrogen dioxide radical ($\cdot NO_2$) (107).

It has long been known that reactive oxygen species such as O_2^- and H_2O_2 produced by the phagocyte oxidase are important components of host defense against a variety of microorganisms. Early experiments by Walker and Lowrie in murine macrophages demonstrated a possible role for ROIs in host defense against mycobacteria, but a large body of work subsequent to this strongly suggests that ROIs have a limited, if any role to play in host defense against tuberculosis (116). ROIs alone may be insufficient to destroy *M. tuberculosis*, but ROI combined with RNIs can significantly enhance mycobacterial killing.

RNIs production by macrophages have long been recognized as important effector mechanism against a variety of pathogens, and RNI in liquid and gaseous form can kill *M. tuberculosis* (190). More recently, accumulating evidence has shown the role of host-derived RNI in the control of tuberculosis (187). In macrophages, NO and other RNIs are derived from L-arginine via an enzymatic pathway controlled by an inducible nitric oxide synthase (NOS2) (215). Studies by Wang *et al.* showed that tubercle bacilli were killed by pulmonary macrophages only if they express NOS2, and this killing could be prevented with a NOS2 inhibitor (307). NOS2 inhibitors were shown to exacerbate the infection in macrophages and in mice treated during either the acute or the chronic phases of the disease (103). The expression of NOS2 and production of potentially mycobactericidal amounts of RNI in murine macrophages and from the lungs of patients with tuberculosis have also been demonstrated (307). Yet the role of

RNI in the antimycobacterial activities of human macrophages still remains controversial.

Various enzymes, peptides, organic acids and lipids present in macrophages have also been reported to be involved in killing of *M. tuberculosis* (218), yet their role in antitubercular activity of macrophages is still a mystery.

Mycobacterial resistance to killing by macrophages

Virulent tubercle bacilli have evolved a wide range of mechanisms to resist host-defense mechanisms. Following attachment and subsequent phagocytosis of *M. tuberculosis*, intracellular mycobacterial growth depends on their ability to avoid destruction by lysosomal enzymes, ROIs, and RNIs present in phagolysosomes of macrophages.

Armstrong and Hart (7) first established that *M. tuberculosis* phagosomal compartments do not mix with ferritin-labeled lysosomes, thus defining the inhibition of phagosome-lysosome fusion. Studies by Gordon *et al.* (135) demonstrated that mycobacteria are capable of producing ammonia, which could both inhibit phagosome-lysosome fusion by alkalinizing the intra-lysosomal contents, and diminishing the potency of the fusion complex. Similarly, sulfatides (derivatives of trehalose 2-sulfate) had been shown to also inhibit phagosome-lysosome fusion (137). More recent studies have confirmed that the *M. tuberculosis* resides in a privileged phagosomal compartment, remaining sequestered away from the terminal endocytic organelles (59, 149, 304, 323). Other studies have shown that *M. tuberculosis* phagosome maturation is

arrested at an early stage characterized by the persistence of early endosomal markers and limited acquisition of late endosomal markers (59, 61, 323). The *M. tuberculosis* phagosome lacks mannose 6-phosphate receptors, exhibits delayed clearance of the major histocompatibility complex (MHC) class I and II molecules, and the endosomal marker, the transferrin receptor (59, 289). These phagosomes appear to acquire limited amounts of major lysosome-associated membrane glycoproteins (CD63, LAMP-1, and LAMP-2) (60, 323) and the lysosomal acid protease cathepsin D (289). Rab5 (small GTP binding protein, which control endocytosis and homotypic fusions within the early endosome) accumulates on the surface of mycobacterial phagosomes, whereas Rab7 (localized to the late endosome) is not associated with the mycobacterial phagosomes (304). These findings demonstrated that *M. tuberculosis* actively retards the maturation of its phagosome at a stage in which the phagosome interacts with early and late endosomes but not with lysosomes.

Importantly, *M. tuberculosis* organisms prevent their phagosomes from developing into acidic hydrolase-rich compartments. The phagosomes containing *M. tuberculosis*, display significantly reduced acidification as compared with killed organisms or model phagosomes containing inanimate objects (323). This has been associated with an apparent paucity or lack of H^+ -ATPase pumps, which are responsible for phagosomal acidification in the vacuolar membranes surrounding the bacilli (289).

Mycobacteria also resist killing by scavenging O_2 with some cell wall components (such as LAM), by inducing detoxifying enzymes (such as catalase, superoxide dismutase, and protective heat-shock proteins), and by resisting RNIs via unknown

mechanisms (218). Mycobacterial LAM interferes with cell signaling pathways, inhibits macrophage activation by INF- γ , and stimulates the production of cytokines such TGF- β and IL-10, which inhibit various macrophage functions (255, 256). Moreover, containment of viable *M. tuberculosis* within these specialized vesicles may reduce the capacity of mycobacterial antigen to be processed, associated with MHC class II proteins, and/or transported to the macrophage cell surface (228).

***M. tuberculosis* induced macrophage apoptosis**

Apoptosis, or programmed cell death, is an evolutionarily conserved, strictly regulated genetic and biochemical program (303) that allows the human body to eliminate unnecessary or damaged cells (264). Apoptosis has been recognized as a component of protective host responses to virus infections for over a decade (58). Target cell apoptosis in response to infections with many intracellular bacteria such as *Shigella* (335), *Salmonella* (54), *Listeria* (142), *Legionella* (206), *Yersinia* (261), *Chlamydia* (221), *Rickettsia* (62), and *Coxiella* (85) has been demonstrated.

Recent studies have provided evidence that alveolar macrophage apoptosis is a common response to intracellular infection by *M. tuberculosis* both *in vitro* and *in vivo* via a TNF- α -dependent pathway (2, 92, 204, 227, 277). Apoptosis of primary human monocyte-derived macrophages following exposure to *M. tuberculosis* H37Rv *in vitro* was reported by Placido *et al.* (242), and Klingler *et al.* (168) noted a similar response to H37Ra. Their results indicate that apoptosis requires challenge with live mycobacteria; heat-killed organisms had no effect on macrophage viability. This

suggests that the signal which primes macrophages for apoptosis is generated from within the cell rather than from activation of a cell surface receptor engaged by bacteria. They also reported that apoptotic macrophages are present in pulmonary granulomas and in bronchoalveolar lavage cells recovered from patients with tuberculosis (168).

The data of Keane *et al.* indicate that TNF- α is a major initiator of apoptotic signaling for macrophages harboring intracellular mycobacteria (166). Infected macrophages become primed for killing by TNF- α , whereas uninfected macrophages are resistant to this effect. In contrast, Fas-mediated death signaling may not play a role in the direct induction of apoptosis of mycobacteria-infected macrophages prior to the recruitment of T cells to the lung when infection is controlled primarily by the innate immune activity of macrophages (219). Fas expression was down-regulated by *M. tuberculosis* and there is no evidence that resting or infected macrophages express FasL (219).

Apoptosis of infected macrophages may contribute to innate immunity to tuberculosis (171). Host protective functions of macrophage apoptosis are likely to include the elimination of a protected intracellular environment conducive to mycobacterial replication or suitable for mycobacterial latency, as well as direct and indirect microbicidal actions (171). It is evident that apoptosis of *M. tuberculosis*-infected macrophages inhibits intracellular bacterial growth and prevents bacterial spread (125, 258). There is evidence that ingestion of bacilli contained in apoptotic cells by freshly added macrophages results in an augmented microbicidal effect (125).

Several studies have observed that the attenuated mycobacterial strains H37Ra and BCG are significantly more potent inducers of apoptosis than virulent strains such as H37Rv, Erdman, and wild type *M. bovis* (124, 167, 173, 212). These results demonstrate that evasion of host alveolar macrophage apoptosis is a *M. tuberculosis* virulence-associated phenotype.

M. tuberculosis has been found to also protect macrophages against apoptosis, either through induction of Toll-like receptor-2 (TLR-2)-dependent activation of the NF- κ B cell survival pathway (13), or by enhancing the production of soluble TNF receptor 2 (sTNFR2), which neutralize the TNF- α (171). However, the role of the anti-apoptotic activities of *M. tuberculosis* in pathogenesis is still not clear.

GENOME SEQUENCE OF *M. TUBERCULOSIS*

The complete genome sequence of the well-characterized H37Rv strain of *M. tuberculosis* was determined in 1998 (66). The genome sequences of a recent clinical isolate *M. tuberculosis* CDC 1551, *M. bovis* BCG Pasteur and *M. bovis* AF2122/97 are nearing completion. The genome of H37Rv comprises 4,411,529 bp and has an average G+C content of 65.6% although some areas with an exceptionally high G+C content (>80%) were detected and found to correspond to a novel PE (ProGlu)-PGRS (polymorphic GC-rich sequence) gene family (247). By means of bioinformatics, 50 genes encoding stable RNA species and 3924 open reading frames (ORF) with the potential to encode proteins were detected (66). These account for >91% of the potential genome coding capacity. The function of about one-third of the protein-

coding genes can be predicted precisely, another third of these gene can be assigned to a functional class with some confidence or have other obvious bacterial homologs (66). The remaining third of the genome, however, encodes proteins we know little or nothing about. These proteins are assigned to the class known as conserved hypotheticals; they are confined to mycobacteria, as they show no similarity to any other reported microbial sequences (65).

On analysis of the genome sequence, no features reminiscent of the horizontally transferred pathogenicity islands as seen in enteric bacteria were found (66). Only a very limited number of genes showing similarity to known virulence genes in other organisms were identified. A homologue of the *Salmonella typhimurium* small protein B gene (*smpB*), whose inactivation in *Salmonella* strains leads to impaired intracellular growth, may be implicated in *in vivo* survival (26, 66). Six of *M. tuberculosis* ORFs with similarity to the gene encoding the p60 secreted virulence factor of *Listeria monocytogenes* could be involved in host cell invasion (66). Four ORFs, which potentially encode phospholipase C, could be virulence factors as in other bacterial pathogens, such as *L. monocytogenes* and *Pseudomonas aeruginosa* (164, 179, 298). Other potential virulence gene homologues identified in the genome sequence include genes with homology to *virF* of *Shigella* (141), putative hemolysin gene *tlyA* of *Serpulina hyodysenteriae* (322), and iron regulation genes (*dtxR*) of *Corynebacterium diphtheriae* (93). However, the role of the above genes in the virulence of *M. tuberculosis* remains to be determined.

VIRULENCE DETERMINANTS OF *M. TUBERCULOSIS*

Virulence genes and Koch's molecular postulates

For many bacterial pathogens, molecular genetic studies over the last two decades have revealed that bacterial disease is a multifaceted phenomenon. Virulence mechanisms can be divided into various steps, each step usually involving various virulence genes rather than a single gene. In the case of *M. tuberculosis*, the steps involved include adherence to and ingestion by macrophages, prevention of phagosome acidification, inhibition of normal phagosome-lysosome fusion, replication within macrophages, spread of infection within the host, and dormancy (107). However, to date mycobacterial genes responsible for these steps remain unknown.

The term “virulence genes” includes all genes contributing to infection as well as disease. To definitely identify sequences as virulence genes, Falkow proposed that a molecular version of Koch's postulates should be met (102). Essentially, the molecular Koch's postulates hold that a virulence gene (and its product) should be found in strains of bacteria that cause a particular disease, but should not be found (or the gene should be present in a mutated form) in strains that are avirulent. Second, disrupting a putative virulence gene in a virulent bacterial strain should reduce the strain's virulence. An alternative to this is that introduction of a putative virulence gene into an avirulent strain should increase the strain's virulence. Third, the putative virulence gene must be expressed by the bacterium at some time during infection of an animal or a human volunteer. Fourth, antibodies against the gene product should be protective or the gene product should elicit a cell-mediate protective immune response (126).

Putative tuberculosis virulence genes

The identification of *M. tuberculosis* virulence genes and gene products is essential to understanding the molecular basis of mycobacterial pathogenesis. However, only a handful of genes from *M. tuberculosis* that fulfill these postulates have been described.

katG

The *katG* gene encodes catalase. It has been known for many years that mutants of *M. tuberculosis* resistant to anti-tuberculous drug isoniazid (INH) were defective for catalase-peroxidase activity (200). Zhang *et al.* (331) first cloned the *katG* gene in the early nineties. They confirmed that the INH resistance phenotype was caused by a mutation in *katG*. Their studies further supported the hypothesis that INH is a prodrug that becomes activated by the catalase-peroxidase (321). The strains of the *M. tuberculosis* complex with little or no catalase activity are resistant to the anti-tuberculous drug isoniazid (INH) and they also have reduced virulence in experimental animals (330). This attenuation is presumably due to their increased sensitivity to the oxidative stresses in macrophages. Wilson *et al.* isolated an INH-resistant, *katG* mutant by exposing a virulent *M. bovis* strain to increasing concentrations of isoniazid (320). This mutant was found to be avirulent for guinea pigs and cattle. With the use of an integrating mycobacterial shuttle vector, they were able to restore a single copy of a wild-type *katG* gene to this mutant. The integration of this gene restored catalase activity as well as INH sensitivity and virulence. This *in vivo* complementation completed the criteria of Koch's molecular postulates and made *katG* the first confirmed tuberculosis virulence gene. More recently, *katG* was also shown to be

important for virulence of *M. tuberculosis* in both guinea pig and mouse infection models (185).

ahpC

In the course of searching for other genes that might be associated with INH resistance, Wilson and Collins isolated an INH-resistant *M. bovis* mutant that had no catalase activity and yet was moderately virulent in guinea pigs (319). This mutant was found to have a mutation in the putative promoter region of *ahpC*, which encodes for alkyl hydroperoxide reductase, and this mutation upregulated the expression of *ahpC*. This upregulation was shown not to confer INH resistance and instead appeared to be a compensating mechanism that enabled strains of the *M. tuberculosis* complex to become INH resistant by losing catalase activity and yet remain virulent. By using an antisense RNA strategy to knock out *ahpC*, Collins' group has shown that the *ahpC* mutant is much less virulent in guinea pigs (317, 318). The functional interactions that occur between *katG* and *ahpC* at the level of protecting against oxidative stress probably reflects the ability of both enzymes to act on one or more common reactive compounds. These may be intracellular organic peroxides, or reactive nitrogen intermediates.

rpoV

A variety of regulation and transcription factors have been shown to play a central role in the virulence of well-studied bacterial pathogens. Collins *et al.* used an

integrating cosmid library of DNA from a virulent *M. bovis* strain to transform an avirulent *M. bovis* to restore virulence (68). Further studies showed that the principal sigma factor gene, *rpoV* (also known as *sigA*), is responsible for this virulence phenotype. A comparison of the DNA sequences of the *rpoV* gene in the virulent *M. bovis* and *M. tuberculosis* and in the avirulent *M. bovis* strain was done. It revealed that loss of virulence in the avirulent strain was due to a single point mutation which caused an arginine-to-histidine change very near a region of the sigma factor that binds to the -35 promoter element (68). This amino acid change in RpoV probably altered its ability to bind an as yet unidentified specific regulatory factor that is required for normal expression of one or more virulence factors. These results suggest that RpoV plays a major role in promoting expression of virulence determinants in member of the *M. tuberculosis* complex (67).

icl

Isocitrate lyase, encoded by the *icl* gene, and malate synthase together form the glyoxylate shunt, which bypasses the CO₂-generating steps of the tricarboxylic acid (TCA) cycle and allows bacteria to synthesize carbohydrates from fatty acids (34). Recent studies revealed that glyoxylate shunt enzymes of *M. tuberculosis* were activated during the metabolic downshift that accompanies oxygen withdrawal in an *in vitro* model of latency (138, 157, 288). Their results suggested that in the absence of an external supply of carbohydrates in lung granulomas, oxygen depletion may trigger mycobacterial adaptation to the latent state. Under these conditions, the glyoxylate

shunt may supply the sugar precursors required for assembly of the carbohydrate-rich mycobacterial cell envelope (217).

Recently, the association of the first enzyme, Icl, with the survival of these persisting organisms has been established. McKinney *et al.* (198) generated an *icl* mutant in virulent *M. tuberculosis* and tested the ability of the wild-type and mutant to infect mice. They found that the mutant had no effect on bacterial number in mice during the acute phase but was cleared from the lungs after two weeks (198). The need for the *icl* gene for mycobacterial survival during late-stage infection indicates that, at two weeks after infection, a change of internal environment may occur that requires the bacilli to alter their diet from carbohydrate to lipid. This shift coincides with the onset of acquired immunity against *M. tuberculosis* and the start of granuloma development. The work of McKinney *et al.* brings into focus another category of *M. tuberculosis* virulence genes — those required for late-stage infection (198). Drugs designed on the basis of the structure of isocitrate lyase might lead to new forms of treatment for latent TB infections.

mce

The *mce* gene was originally described as encoding a putative mycobacterial cell entry protein. Arruda *et al.* (8) used a non-invasive strain of *E. coli* as a surrogate host and screened a genomic *M. tuberculosis* library prepared from the avirulent strain H37Ra. They isolated a DNA fragment responsible for the ability of *E. coli* to bind and to subsequently invade non-phagocytic HeLa cells and identified the *mce* gene as

conferring the phenotype (8). The DNA fragment appeared to be sufficient to increase the uptake of recombinant clones by macrophages and to prolong their intracellular survival. Moreover, a recent study showed that a *mce* knock-out mutant exhibits a reduced ability to invade non-phagocytic cells (119). Genome sequence analysis revealed that there are 4 *mce* genes arranged into four separate operons composed of 5 additional genes at multiple sites of *M. tuberculosis* genome (65). Genes preceeding *mce* all contain the RGD tri-peptide motif that has been shown to be involved in receptor attachment, while the following five genes are all predicted to encode proteins which are either secreted or surface-exposed (65). These results support the proposed role of Mce in the invasion of host cells by *M. tuberculosis*. However, deciphering the exact role of the *mce* operon family in virulence will be complicated by the presence of four operons, as inactivation of one may be compensated for by any of other three copies.

erp

The *erp* gene encodes the exported repetitive protein (33). It is found exclusively in slow-growing mycobacteria causing tuberculosis and leprosy (33). Erp contains a typical N-terminal signal sequence, a hydrophobic domain at the C-terminus and it harbours repeated amino acid motifs, suggesting it is a cell-wall-associated surface protein. This gene was inactivated in both *M. tuberculosis* and *M. bovis* BCG by allelic replacement with a mutated *erp* gene that was disrupted by insertion of a cassette containing a kanamycin resistance gene (32). The resulting *erp* mutant strain was found

to be less virulent in mice and showed impaired growth in macrophages cultured *in vitro*. Complementation with the intact gene restored the wild-type phenotype (32). These results suggest that the *erp* gene, by some unknown mechanism, contributes to the virulence of mycobacteria.

acr

The *acr* gene encodes α -crystallin, sometimes called the 16-kDa antigen. The Acr protein is recognized by the immune response of patients with tuberculosis (97). Studies by Barry and colleagues (325) using 2-D protein gels demonstrated that at least six proteins are up-regulated in stationary-phase cultures of *M. tuberculosis*. One of the most prominent stationary phase-induced proteins is α -crystallin, which is a chaperonin that assists in protein folding and protects other cellular proteins from degradation (325). To determine the role of Acr in *M. tuberculosis* survival within macrophages, Yuan *et al.* (326) constructed an *acr* mutant by allelic exchange to inactivate the *acr* gene, and found that its disruption impairs the ability of the mutant to grow in the THP-1 macrophage cell line (326). These results suggest that the Acr protein plays an important role in facilitating survival of *M. tuberculosis* during tuberculosis infection.

TECHNIQUES FOR IDENTIFYING CANDIDATE VIRULENCE FACTOR

Expression of mycobacterial genes in surrogate bacterial hosts

The recent development of techniques for the genetic manipulation of *M. tuberculosis* has enhanced our ability to identify genes that play a role in microbial

pathogenesis. As demonstrated in several bacterial genera, virulence factors can be identified by transferring cloned DNA from a pathogenic strain to (i) a closely related non-pathogenic species, (ii) a distant genus that can efficiently express the transferred genes, or (iii) a mutant with attenuated virulence, and then screening for the acquisition of a specific pathogenic trait (151). The studies of mycobacterial gene expression in surrogate hosts have served to identify several genes that may very well be involved in the pathogenesis of mycobacteria.

The rapidly-growing nonpathogenic *M. smegmatis* and *E. coli* have been used as surrogate host strains to identify genes from a virulent *M. tuberculosis* strain that enhance survival in macrophages. Arruda *et al.* used a non-invasive strain of *E. coli* as a surrogate host to identify a DNA fragment responsible for the ability of *E. coli* to bind and subsequently invade HeLa cells, leading to the identification of the *mce* gene (8).

Ehrt *et al.* constructed an *M. tuberculosis* genomic library and screened it in *E. coli* under acidified nitrate conditions to select for clones expressing RNI resistance (95). They identified the *noxR1* gene that confers resistance to both RNIs and to ROIs upon both *E. coli* and *M. smegmatis* in culture and within macrophages. Ruan *et al.* identified another potential RNI resistance gene of *M. tuberculosis*, *noxR3*, by screening an *M. tuberculosis* library using a *Salmonella typhimurium* system (260). Schinnick and associates screened a cosmid *M. tuberculosis* DNA library in *M. smegmatis*, using a human macrophage-like cell-line. They identified several potential genes that enhanced *M. smegmatis* intracellular survival (207).

In other studies, attenuated strains of *M. tuberculosis* complex species were also used as surrogate hosts. Collins *et al.* screened a virulent *M. bovis* cosmid library in an attenuated *M. bovis* strain by passage through guinea pigs (68). By this procedure, they identified the *rpoV* gene which could restore virulence to the attenuated *M. bovis* strain. Pascopella *et al.* used a cosmid library of virulent H37Rv to transform avirulent H37Ra and screened the library by passage through mice for recombinants that were better able to grow and persist in the spleen and lungs (232, 233). They identified a 25-kb DNA fragment, *ivg*, which conferred enhanced *in vivo* growth to avirulent H37Ra (233).

However, as noted above, pathogenesis is a multifaceted phenomenon, involving the expression of numerous genes. It is unlikely that all of the requisite genes will be located in close proximity on the mycobacterial chromosome and therefore be introduced simultaneously into the surrogate host. Although the cloned genes may be expressed in a surrogate host such as *M. smegmatis*, the products of individual genes may not be sufficient to permit survival and multiplication within infected mammalian cells.

Transposon mutagenesis

Transposon mutagenesis is an excellent method of producing libraries of bacterial mutants by using mobile insertion elements that randomly disrupt chromosomal genes. In 1990, derivatives of IS6100, an insertion sequence from *M. fortuitum*, were used to demonstrate transposition in mycobacteria and were delivered on thermosensitive

vectors into *M. smegmatis* to create the first mycobacterial insertional mutant libraries (195). McAdam *et al.* constructed a suicide transposon-containing plasmid library generating a moderate numbers of mutants. By using this system, three auxotrophs, two for leucine and one for methionine were isolated in *M. bovis* BCG (196).

In order to identify potentially avirulent strains, more efficient systems based on phage delivery (15) and a thermosensitive plasmid harbouring *sacB* as a counterselectable marker have been developed (140). These systems are being used to produce large numbers of mutants that will be subsequently screened for such properties as auxotrophy or the ability to survive within macrophages. Transposon libraries have now been made in *M. smegmatis*, *M. bovis* BCG, and different virulent strains of *M. tuberculosis* (15, 140, 196). The random nature of the transposon insertion has been confirmed by DNA sequencing (15). Jackson *et al.* recently constructed a transposon library in a clinical strain (Mt103) of *M. tuberculosis* and successfully isolated a mutation in the gene encoding antigen 85C, *fbpC* (161). This mutant had a reduced ability to transfer mycolates into the cell wall and demonstrated that activity of antigen 85C is not specific to the mycolate type.

One of the strengths of transposon mutagenesis is that no previous assumptions are made about the identity of the gene responsible for a particular virulent trait. But its major limitation is the availability of an appropriate screening system for the large numbers of mutants produced. One way to circumvent this problem is to adapt these transposon systems to a signature-tagged mutagenesis method as described below.

Subtractive techniques

Differentially expressed genes may be identified as genes that are expressed by one strain and not by another closely related strain, or that are expressed by an organism in response to stimuli from one environment but not from another. In the field of mycobacterial research, DNA or cDNA subtractive techniques and differential display techniques have been employed to study differential gene expression.

DNA subtractive hybridization was used to identify some gross genetic differences between *M. bovis*, *M. bovis* BCG, and *M. tuberculosis* (191). Three large deletions (RD1, RD2 and RD3), representing ~30-kb of DNA, were found in BCG. RD3 corresponds to a region containing major immunogenic antigens (such as ESAT6, MPB64) and is deleted in some strains of *M. bovis* BCG (191). The RD1 region is deleted in all strains of *M. bovis* BCG. Using BAC array (ordered overlapping cosmid hybridization), Gordon *et al.* identified more regions (RD4 – RD10) of *M. tuberculosis* DNA absent from *M. bovis* (136). Some of these genes may play a role in determining the different host specificities of *M. tuberculosis* and *M. bovis*.

In cDNA subtractive hybridization, mycobacterial mRNA is reverse transcribed into cDNA which is then used for hybridization. Kinger *et al.* first reported the use of cDNA subtractive hybridization in mycobacteria by analyzing the differences in gene expression between *M. tuberculosis* H37Rv and H37Ra (168). Five mRNAs were found preferentially expressed in H37Rv. Yet no sequence information was available and the corresponding genes remain uncharacterized. Plum *et al.* reported a cDNA subtraction hybridization technique using different libraries constructed of cDNA from

a single strain of *M. avium* grown either in liquid culture or in a macrophage culture assay (244). A macrophage-induced gene, *mig*, was identified which encodes a predicted 27-kDa exported protein of unknown function (244). The *mig* gene is absent from the *M. tuberculosis* genome, thus emphasizing the different mechanisms of pathogenesis in these two mycobacteria species. Recently, Clark-Curtiss and her associates have developed a selective capture of transcribed sequences (SCOTS) method to identify genes that are expressed by *M. tuberculosis* in macrophages but not when the organisms are cultured *in vitro* (138).

Differential display techniques have also been used to identify genes that are expressed in *M. tuberculosis* H37Rv but not in H37Ra (253, 254). A complementary approach to these nucleic acid techniques is proteomic comparison. In this approach, differences are detected on two-dimensional gels between proteins from a virulent and an avirulent strain or between proteins from one strain grown under different conditions (259, 288, 296). But further genetic studies are essential to determine the role in virulence of individual genes and their protein products with such an approach.

Perhaps the most interesting facet of subtractive hybridization is that differentially expressed genes can be identified directly from clinical specimens. In addition, this type of procedure would help avoid the debate over the most appropriate virulence model to use for a pathogen. Limitations of this technique include the requirement for large numbers of starting bacteria and separation of bacterial RNA from contaminating host RNA (87). But these technical problems will be overcome as the RNA isolation and bacterial reverse transcription technologies improve and become more sensitive.

Gene fusion

Fusions to promoterless reporter genes, whose expression can be easily monitored, have been widely used to identify potential virulence factors in pathogenic bacteria. The reporter genes that have been demonstrated to function in mycobacteria (*phoA*, *lacZ*, *lux*, *xylE* and *gfp*) have been mainly used to study gene expression, leading to a better knowledge of the mycobacterial transcription machinery (151). β -galactosidase (LacZ) is one of the most widely used reporters of bacterial gene expression (123). The *E. coli lacZ* gene is expressed in mycobacteria when fused to a mycobacterial promoter, and β -galactosidase activity can easily be assayed for in cell extracts or intact mycobacterial cells (209, 283). The use of *lacZ* fusions enables one to estimate the activities of promoters during the intracellular growth of mycobacteria within macrophages. Dellagostin *et al.* demonstrated that the promoter of a 18-kDa gene of *M. leprae* is specifically activated during intracellular growth using a translational fusion with *lacZ* (86). However, the instability of LacZ in mycobacteria, particularly in *M. smegmatis*, hampers its application (175).

The *phoA* gene encodes alkaline phosphatase, an enzyme that must be exported across the cytoplasmic membrane to be active and whose activity can be monitored by a simple plate-based assay (186). Translational fusions with PhoA will allow the identification of potential mycobacterial virulence factors that are either secreted or surface-associated. The utility of *phoA* as a reporter gene for studying protein export in mycobacteria was demonstrated by using *blaF-phoA* gene fusions (297). Using a genomic library of *M. tuberculosis* DNA fused to a promoterless *phoA* gene, numerous

DNA inserts (including *erp*) that direct the production and export of PhoA have been identified (186). A further detailed characterization of these proteins will help to evaluate their involvement in mycobacterial virulence and protective immunity.

The *gfp* gene, encoding the green fluorescent protein (GFP), appears more useful for *in vivo* studies because no substrate is required for detection of its production. Dhandayuthapani *et al.* demonstrated the GFP could be used for analysis of mycobacterial gene expression *in vivo* to study mycobacteria-macrophage interactions (88). The use of GFP fusions in *M. marinum* identified several promoters that are induced in macrophages (16, 17). A variety of mycobacterial promoters from genes such as *ahpC*, *hsp60*, and *mtrA* fused to *gfp* were studied by different fluorescence and microscopic techniques (88, 172). Fluorescent recombinant clones that produce the GFP in response to a defined stimulus can be sorted from a population of bacteria using fluorescence activated cell sorter (FACS) analysis (73). Using recombinant *M. bovis* BCG expressing *gfp-mrt* fusions, phagocytic vesicles containing mycobacteria were isolated. Subsequently, GFP-tagged *M. avium* was visualized inside human macrophages and epithelial cells by fluorescence microscopy (230). GFP was used to identify the stage in macrophage infection when the fusion of phagosomes containing mycobacteria to lysosomes gets arrested and to study the effect of proinflammatory and anti-inflammatory cytokines on the maturation of mycobacterial phagosomes (304). A vector containing GFP was used to construct a promoter library of *M. tuberculosis* in *M. bovis* BCG to find genes that are upregulated after infection of macrophages (301). Seven genes were identified, including *fadB4*, *cysD*, *sseA*, and *moeZ*, which were

activated within phagocytes. The study of mutant strains in which these genes are inactivated will provide information on their role in intracellular survival.

Recent techniques with potential application to identification of virulence factors in *M. tuberculosis*

In the last 5 years, major progress in global investigation methods and genetic tools have opened the way for the studying of pathogenicity during infection and survival in the host. Application of new techniques such as *in vivo* expression technologies, signature-tagged mutagenesis, and microarrays are expected to provide a strong basis for studying *in vivo* induced genes, and for a better understanding of mycobacterial pathogenicity.

***In vitro* expression technology (IVET)**

The IVET technique was originally described for *S. typhimurium* based on the use of *lacZ* as the reporter gene (192). In the IVET system, a promoter fusion library is constructed in which cloned promoters direct expression of a gene that complements a defect in an auxotrophic mutant of the host strain (144, 182). The desired fusion will only or preferentially express the complementing marker under proper *in vivo* conditions. With the improvement for enhancing DNA recombination and integration, and the advent of selectable and auxotrophic makers (15, 196), it is now possible to apply IVET to mycobacteria. Recently, Smith *et al.* have employed an isoniazid-resistance-based vector in an IVET study (279). By screening for *M. tuberculosis*

promoters that confer resistance to isoniazid in macrophages or mice, several genes were identified, including those encoding an ESAT-6-like protein and an enzyme involved in carbohydrate metabolism (279).

Signature-tagged mutagenesis (STM)

STM is a recently developed technique that combines the power of insertional mutagenesis with *in vivo* negative selection using a limited number of animals, to identify attenuated derivatives from a complex pool of transposon mutants (152). The unique signature tag in each mutant allows identification of attenuated mutants that do not survive the selective process. It has been successfully applied to identify virulence genes in various pathogens such as *Salmonella typhimurium* (57), *Vibrio cholerae* (57), *Staphylococcus aureus* (199), and *Legionella* (94). Several transposon-mediated mutagenesis systems available for mycobacteria could be adapted for STM. To identify the genes required for the pathogenicity of *M. tuberculosis*, Guilhot's group recently constructed a library of signature-tagged transposon mutants of *M. tuberculosis* and screened for mutants unable to multiply within the lungs of mice (47). Sixteen of the 1927 mutants identified were attenuated for virulence, and most of the mutated loci appeared to be involved in lipid metabolism or transport across the bacterial membrane. Four independent mutations were identified as a cluster of virulence genes located on a 50-kb chromosomal region (47). These genes were found involved in the production of phthiocerol and phenolphthiocerol derivatives. The products of these genes might be restricted to pathogenic mycobacterial species. A second independent study with STM

identified three mutations in genes involved in virulence in the same 50-kb region: two mutants have transposon insertions affecting genes implicated in phthiocerol dimycocerosate (PDIM) synthesis; the third has a disruption in a gene encoding a large transmembrane protein required for proper subcellular localization of PDIM (74). These results showed that synthesis and transport of this complex lipid is only required for growth in the lungs but is not required for growth in the liver and spleen.

The selection scheme in these studies (such as the animal model, the route of infection, the size of the infection dose, the target organ, and the time post infection used) might limit the number of genes identified to those required for the initial multiplication within lungs (239). Yet, the adaptation of the STM method to *M. tuberculosis* opens the way to a broader use of this procedure to target genes important in other steps of the mycobacterial infection process.

Microarray technology

Microarray assays are based on the ability of labeled cDNA sequences from cells or tissues (probes) to hybridize to complementary sequences (targets) immobilized to solid surfaces (96). The real power of microarrays lies in generating biological hypotheses, pointing the way towards unexpected or unpredicted relationships between diverse sets of genes on the chromosome and suggesting a functional role for proteins that have no known homologs (44, 91). The determination of the genome sequence of *M. tuberculosis* H37Rv provides a framework for the microarray genomic analysis of H37Rv and other members of the *M. tuberculosis* complex (66). To establish which

regions of the *M. tuberculosis* genome were missing from a collection of BCG daughter strains, Behr *et al.* used a DNA microarray representing 99.4% of open reading frames (ORFs) of H37Rv to perform parallel comparative hybridizations between *M. tuberculosis* H37Rv and *M. bovis* BCG strains (28). Their results showed that 16 deleted regions which corresponded to 129 ORFs were absent in BCG strains. Although the role of this collection of ORFs absent from *M. bovis* isolates remains to be determined in *M. tuberculosis*, it is tempting to speculate that some of them may serve a role in adapting to environmental change, and may be involved in the progressive attenuation of virulence. Wilson and co-workers used a DNA array containing 97% of the ORFs predicted from the genome of *M. tuberculosis* to monitor changes of gene expression in response to treatment with the anti-tuberculosis drug isoniazid (316). Their results showed that isoniazid induced several genes that encode proteins physiologically relevant to the drug's mode of action.

While this technology holds promise, microarray hybridizations have certain limitations. First, they are not widely available and require significant investment in equipment. Second, they display a fairly small dynamic range of induction or repression when used in different modes. Lastly, poorly expressed genes are not likely to be detected by microarrays and subtle changes in rare transcripts that encode important regulatory proteins could easily escape notice (22). As the technology advances, it is anticipated that more efficient hybridization, lower background, and better signal to noise ratios will increase the sampling efficiency of genes encoded by *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1 and Table 2, respectively. *E. coli* DH5 α was used as the host for all plasmid constructions. It was grown on Luria–Bertani (LB) medium supplemented with antibiotics. *M. smegmatis* 1-2c (281) was used as a host strain to screen and evaluate mycobacterial genes. It was grown in Middlebrook 7H9 (Difco, Detroit, MI) liquid medium and on Middlebrook 7H10 (Difco) agar. *M. tuberculosis* strains H37Ra and H37Rv were grown on Middlebrook 7H9 or 7H10 supplemented with albumin dextrose complex (ADC) (0.5 % bovine serum albumin (BSA), 2 % dextrose, and 0.085 % NaCl) or OADC (0.005 % oleic acid, 0.2 % dextrose, 0.5 % BSA and 0.085 % NaCl) supplemented with 1 % glycerol and 0.05% Tween 80 at 37°C for 14 to 21 days. For *E. coli*, antibiotics (Sigma, St. Louis, MO) were used at following concentrations: ampicillin (Amp) 100 μ g/ml; kanamycin (Kan) 50 μ g/ml; hygromycin B (Hyg) 200 μ g/ml. For mycobacteria, antibiotics were supplemented at the following concentrations: gentamicin 10 μ g/ml, hygromycin B 50 μ g/ml, and kanamycin 25 μ g/ml. All work with virulent *M. tuberculosis* strain H37Rv was done in a Bio-Safety Level 3 (BSL3) facility.

In vitro growth studies

In vitro growth rates of the different *M. tuberculosis* H37Ra/H37Rv (wild-type) strains and the isogenic Δ *eis* mutants were determined in agitated cultures grown at 37

°C either in enriched Middlebrook 7H9 with ADC or in synthetic glycerol-alanine-salts (GAS) medium (84). Each 50-ml culture was started by inoculation with 100 µl of a mid-log phase (2-week) culture. Aliquots were sampled every 1 to 2 d, and the bacterial density was determined by measuring the OD₆₅₀ using a Bio-Rad SmartSpec 3000 (Bio-Rad, Hercules, CA).

General DNA methods

For DNA manipulations, standard protocols were followed (263). Agarose was purchase from Promega (Madison, WI). Restriction enzymes, Klenow fragment of T4 DNA polymerase, T4 DNA ligase, and calf intestinal alkaline phosphatase (CIAP) were from New England BioLabs (Beverly, MA) or Gibco BRL Life Technologies (Gaithersburg, MD). All reagents were used according to the manufacturer's instructions. Plasmids were constructed in *E. coli* HB101 or DH5α cells, and prepared using a Qiaprep spin miniprep kit (Qiagen, Valencia, CA).

Mycobacterial genomic DNA preparation

To construct the genomic library or for Southern blot analysis, chromosomal DNA of *M. tuberculosis* H37Rv or H37Ra was isolated by the method of Bose *et al.* (41). *M. tuberculosis* strains were grown in 50 ml liquid 7H9 medium supplemented with ADC and 1% glycerol for two weeks to reach the middle exponential phase (OD₆₅₀ 0.5 –1.0). Cells were pelleted (~100 mg wet weight) and washed twice with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). One and half milliliter of saturated cesium chloride solution

containing 1% Triton-X 100 was added and then the mixture was rocked manually to prepare a thorough suspension. Then 45 ml of distilled H₂O was added to the cell suspension to generate osmotic shock and burst the tough mycobacterial cell wall. Cells were centrifuged and resuspended in 2.5 ml of TE buffer containing fresh lysozyme (1mg/ml) and kept on ice for 1 h. Ten percent SDS (to final concentration 0.5 %) and proteinase K (to final concentration 100 µg/ml) were added and mixtures were placed in a 55 °C water bath for 4 h. Then 2.5 ml of 4 M GIT Cocktail (0.5 g/ml guanidium thiocyanate, 53 mM Tris-HCl (pH 7.5), 12 mM EDTA (pH 8.0), 0.2M NaCl, 0.15M 2-mercapto-ethanol, 2.12 % N-lauroyl sarcosine) and 10% CTAB (cetyl trimethyl ammonium bromide to final concentration 1 %) were added. The mixtures were slowly shaken for 30 min in a 65°C water bath to remove the bound polysaccharides. An equal volume of 25:24:1 phenol /chloroform /isoamyl alcohol was then added, mixed well, and centrifuged at 5,000 rpm for 5 min to separate phases. The upper aqueous phase was transferred to a new centrifuge tube and a half volume of 5 M ammonium acetate and 0.75 volume of isopropanol were added and the sample allowed to sit at – 20 °C overnight. The next day the tube was microcentrifuged in cold for 10 min, DNA pellets dried, and resuspended in 500 µl TE (pH 7.5).

Construction of an *M. tuberculosis* H37Rv genomic plasmid DNA library

Genomic DNA was isolated from the virulent strain of *M. tuberculosis* H37Rv as described previously (331). In brief, fresh 7H9 liquid cultures of H37Rv were inoculated with growth from a 3-week starter culture and then grown for 7 days before

harvesting. H37Rv genomic DNA was isolated and then partially digested with the restriction enzyme *Sau3AI*, and 4- to 12-kb fragments were recovered after electrophoresis in a 0.7% agarose gel. DNA was extracted from the gel and cloned into the *Bam*HI site of the plasmid pOLYG (133, 311). The library DNA was transformed into Max Efficient™ *E. coli* DH5α (Gibco BRL) competent cells, plated on LB agar containing 200 µg/ml hygromycin B, and incubated overnight at 37 °C. Approximately 7,000 independent *E. coli* transformants were generated by using this plasmid library. Restriction analysis of plasmid isolated from 16 of the resultant colonies demonstrated that 81% had insert DNA with an average insert size of 5.4-kb. This library pool was mixed in LB-30% glycerol, and aliquots were frozen at –70 °C. *E. coli* DH5α containing the *M. tuberculosis* plasmid library was amplified by growth on LB plates, and plasmids were isolated by following the Qiagen Maxi-plasmid purification protocol. The isolated plasmid library DNA was then used to transform *M. smegmatis*.

Electroporation of mycobacteria and *E. coli*

M. smegmatis was prepared for electroporation as previously described (281). Briefly, a 50-ml culture of *M. smegmatis* 1-2c was grown in Middlebrook 7H9 broth with gentle shaking for 3 d to mid-log phase ($OD_{650} = 0.5-1.0$). The bacterial culture was incubated on ice for 10 minutes, transferred to 50-ml chilled conical tubes, and washed three times with $1/2$ to $1/50$ volume of cold 10% glycerol. The cell pellets were resuspended in 5 ml of cold 10% glycerol and stored at –70 °C until use. Aliquots of DNA (1 to 10 µg) and 400 µl of fresh prepared electrocompetent cell suspension were

mixed gently in a prechilled GenePulser electroporation cuvette with 0.4-cm-gap-size cuvette (Bio-Rad) and pulsed twice with GenePulser apparatus (Bio-Rad) at settings of voltage 2.5 kV, capacitance 25 μ F, and resistance 1,000 ohms. Six hundred microliters of 7H9 broth without antibiotics was added immediately, the cell suspension was transferred to a snap-cap tube (Falcon) and incubated at 37 °C with agitation for 3 h. Transformants were then plated on 7H10 plates containing the antibiotic of choice (kanamycin at 25 μ g/ml or hygromycin B at 50 μ g/ml), and incubated at 37 °C for 3 to 4 d. Individual antibiotic-resistant colonies were subcultured onto fresh 7H10-antibiotics plates and grown an additional 3 to 4 days prior to further evaluation. *M. tuberculosis* strains were electroporated almost the same way as described for *M. smegmatis*, except all manipulation were done at room temperature instead of on ice. The expression step proceeded overnight for approximately 12 h prior to plating on 7H10-OADC-Tween plates with incubation at 37 °C for 3 to 4 weeks.

To analyze the recombinant clones, plasmid DNA was electroporated directly from recombinant *M. smegmatis* to *E. coli* cells. Electrocompetent *E. coli* cells were prepared as previously described (263). Sixty microliters of electrocompetent *E. coli* cells were pipetted into a 0.1-cm-gap-size cuvette containing 40 μ l of transformed *M. smegmatis* cells. Electroporation was performed at settings of voltage 2.5 V, resistance 200 Ω , and capacitance 25 μ F.

DNA sequencing and analysis

DNA sequencing was performed at the Laboratory of Molecular Systematics and

Evolution, University of Arizona Division of Biotechnology (Tucson, AZ), using a DNA sequencer (Applied BioSystems Model 377A). Sequences were analyzed using GCG sequence analysis programs (Version 10, Genetics Computer Group, University of Wisconsin, Madison, WI) and the National Center for Biotechnology Information BLAST network service. Primers for sequencing were synthesized by Gibco BRL Life Technologies (Table 3).

Polymerase chain reaction (PCR)

Primers used in this study for PCR analysis are listed in Table 3. All primers were synthesized by Gibco BRL Life Technologies. PCR amplifications were performed using a 2400 GeneAmp PCR system (Perkin-Elmer, Foster City, CA). PCR conditions were 10 mM Tris (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X100, 10% DMSO, 200 μM deoxynucleoside triphosphates (dNTPs) (Boehringer Mannheim), 0.5 μM of each primer, and 2.5 U of *Taq* polymerase (Life Technologies) per 50 μl of reaction mixture. The PCR programs used were as follows: 1 cycle of 94°C for 5 min; 25 cycles of denaturing at 94°C for 1 minutes, annealing at 56°C for 1 min; and a final cycle of extension at 72°C for 10 min. PCR products were resolved in a 0.7% agarose gel and stained with ethidium bromide.

Construction of *eis* deletion derivatives

The DNA insert of p69 was released from pOLYG by digestion with *Bam*HI and cut in the middle of open reading frame 2 (ORF2) (Figure 8) by digestion with *Sph*I.

The resulting 1.3- and 1.7-kb fragments were ligated into *Bam*HI-*Sph*I-digested pUC18 to yield the subclones p62-8 and p62-16, respectively. The 1.3-kb *Bam*HI-*Hind*III fragment from p62-8 was cloned into *Bam*HI-*Hind*III-digested pOLYG to obtain p69-8. The 1.7-kb *Bam*HI-*Hind*III fragment from p62-16 was then cloned into *Bam*HI-*Hind*III-digested pOLYG to obtain p69-16. p62-97 was created by digestion of p69 with *Apo*I and *Pvu*I. The *Pvu*I site was filled in with DNA polymerase. This 1.6-kb *Apo*I-*Pvu*I fragment carrying only ORF2 and its putative promoter region was isolated and subcloned into *Eco*RI-*Sma*I-digested pSP72 (Promega). p69-97 was constructed by inserting the 1.6-kb *Eco*RV-*Hind*III fragment from p62-97 into *Eco*RV-*Hind*III-digested pOLYG. p69-96 was made by inserting the 1.6-kb *Cla*I-*Hind*III fragment from p62-97 into *Cla*I-*Hind*III-digested pOLYG. The transcriptional orientation of p69-96 is opposite that of p69-97. ORF2 was disrupted by removing an internal 367-bp *Apa*I fragment from p69-96, creating p69-96A, which is an in-frame deletion. All p69 derivatives and p69 itself were then retransformed into *M. smegmatis* cells for use in the intracellular survival assay.

Construction of *M. smegmatis* with a single chromosomal copy of *eis*

Insertion of *eis* in a single copy into the chromosome of *M. smegmatis* was carried out using the procedure described by Lee *et al.* using the integrative plasmid vector pMV306 (181). The *eis* gene was cloned into this integrating vector by inserting the 1.6-kb *Cla*I-*Hind*III fragment from p62-97 into *Cla*I-*Hind*III-digested pMV306. The recombinant plasmid was electroporated into electrocompetent *M. smegmatis* 1-2c as

described previously. The vector backbone of pMV306 contains a kanamycin resistance cassette but does not contain a mycobacterial origin of replication and therefore can be maintained only by integration into the host chromosome.

Transformants were selected by growth on 7H10 plates with kanamycin (25µg/ml). The presence of *eis* in the *M. smegmatis* chromosome was validated by Southern hybridization using DIG-labeled *eis*-probe 1 (Table 3). The chromosomal DNA was isolated from potential clones, digested with *Cla*I and *Hind*III for Southern analysis.

Construction of a plasmid for *eis* gene disruption

The plasmid p6297 that contains *eis* and its putative promoter region in a 1.5-kb *Cla*I-*Hind*III fragment was used for allelic replacement. The *eis* gene was disrupted by removing a 367-bp intragenic *Nhe*I fragment with the insertion of a 1.3-kb *Xba*I released cassette carrying the *Streptomyces hygroscopicus* hygromycin B gene (*hyg*) gene (327) from pRL498 (a gift from Clifton Barry III). This generated p6237 with a 2.5-kb insert (*eis*::*hyg*) which contained 475-bp of *eis* left-flanking DNA and 520-bp of *eis* right-flanking sequences around a central *hyg*. The 2.5-kb *Cla*I-*Hind*III fragment excised from p6237 was cloned into *Cla*I-*Hind*III-digested pGEM-7Zf(+) to generate p6239. The 2.5-kb *eis*::*hyg* allele was then released from p6239 with *Xba*I-*Bam*HI and cloned into *Xba*I-*Bam*HI-digested suicide vector pMJ10 (237), generating the disruption construct p6308.

Allelic replacement and complementation of the *eis* gene in *M. tuberculosis*

Five micrograms of p6308 was introduced into freshly prepared electrocompetent *M. tuberculosis* strain H37Rv or H37Ra as described previously. Transformants were selected on 7H10-OADC-Tween 80 plates containing hygromycin B at 37 °C for 3 to 4 weeks. Individual antibiotic-resistant colonies were subcultured onto fresh 7H10-OADC-antibiotics plates and grown an additional 2 to 3 weeks prior to preparation of frozen stocks and further evaluation.

A few clones were randomly selected and grown in 50 ml of 7H9-ADC broth with hygromycin B at 37 °C for 48 h. These liquid cultures were then diluted with 7H9-ADC broth (10^{-3} to 10^{-8}), the dilutions plated on 2% sucrose-7H10-OADC-hyg plates and plates incubated at 39 °C for 3 to 4 weeks. The double-resistant ($\text{suc}^R/\text{hyg}^R$) clones were picked to further evaluate the inactivation of *eis* gene by Southern blot analysis.

Once the *eis* knock-out (Δeis) construction was confirmed, the integrative single-copy-*eis*-bearing plasmid p6301 was used for complementation of the *M. tuberculosis* Δeis mutant. Construct p6301 was introduced into *M. tuberculosis* Δeis mutant by electroporation and kanamycin selection. Southern blot analysis was used to confirm the chromosomal structure of candidate knock-out and complementation strains by using a DIG-labeled *eis*-probe 2 (Table 3 and Figure 9). The restriction enzyme *Xho*I was used to digest chromosomal DNA isolated from candidate clones. Western blot analysis using anti-Eis antibody was also performed for the detection of the production of Eis protein by wild-type, candidate knock-out, and complementation strains (Table I).

Southern hybridization

Southern hybridizations were performed using a DIG-DNA labeling and detection kit (Boehringer Mannheim) (9). Chromosomal DNA was digested with the appropriate restriction enzyme and electrophoresed through a 0.8 % agarose gel. The gel was incubated in 0.25 N HCl for 15 min, neutralized in 0.4 M NaOH, and 0.6 M NaCl for 30 min. The DNA was transferred to a GeneScreen Plus membrane (NEN Research Products, Boston, MA) by using a Vacublot Transfer Apparatus (American Bionetics, Hayward, CA), and the membrane was dried at 37°C for 15 min. The membrane was incubated for 1 h at 65°C in prehybridization solution (0.5% blocking reagent (Boehringer Mannheim), 0.02% SDS, 0.75 M NaCl, 75 mM sodium citrate (pH 7.0), 0.1 % N-lauroylsarcosine) and then in fresh prehybridization solution containing 10-20 ng/ml of the appropriate DIG-labeled DNA probe overnight at 65°C in a heat sealable plastic pouch (KAPAK, Minneapolis, MN). The membrane was then washed four times (15 min per wash) at 65°C in 0.3 X SSC (45 mM NaCl, 4.5 mM sodium citrate, pH 7.0), 1.0 % SDS, followed by detection of digoxigenin-labelled fragments according to the DIG-DNA labeling and detection kit manual. Bands were detected using anti-digoxigenin-alkaline phosphatase-conjugated antibodies and nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (BCIP) (Boehringer Mannheim) as substrates.

Two DNA probes (*eis*-1 probe and *eis*-2 probe) were used in Southern blot analysis (Table 3). The *eis*-1 probe was used in studies for detection of the presence of *eis* in mycobacteria. The *eis*-2 probe was used for confirming *eis* knock-out mutants. These DNA probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) using

PCR. The 5' primer for *eis-1* probe was 69.4F and the 3' primer for *eis-1* probe was 69.8R; the 5' primer for *eis-2* probe was 69, and the 3' primer for *eis-2* probe was *eis-dig-r* (Table 3).

Preparation of human monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by centrifugation on Ficoll-hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) as described previously (42, 128). Briefly, fifty milliliters of blood was diluted 1:1 with warm Hbss buffer, layered on Ficoll-hypaque, and centrifuged at 2,000 rpm for 40 min in a Beckman Model T J-6R centrifuge. After the centrifugation, the total leukocyte fraction was collected and washed with 10 ml of Hbss buffer at 1,500 rpm for 5 min. The cells were resuspended in RPMI 1640 (Sigma, St. Louis, MO) containing 15 % autologous human serum and adjusted to 10^6 cells per ml. Appropriate volumes of mononuclear cells (to give 2×10^5 monocytes per well) were incubated in wells of a 24-well tissue culture plate (Costar, Cambridge, MA). After 2 h incubation at 37 °C in a CO₂ incubator, cells were washed three times with warm RPMI-1 % autologous serum to remove nonadherent cells. Adherent cells were 90 to 95% monocytes, as verified by Giemsa stain. Adherent cells were cultured in 1 ml of RPMI-15 % autologous serum at 37 °C with 5 % CO₂ overnight to allow maturing into macrophages prior to the infection. The number of adherent cells per well was determined by counting nuclei after lysis of the monolayer (213). At this point, each well contained approximately 2 to 4×10^5 cells. Normally, macrophages and serum

from the same donor were used.

Intracellular survival assay

The intracellular survival assay was devised by modifying the procedure of Ramakrishnan and Falkow (248) developed for *Mycobacterium marinum* and the mouse macrophage-like cell line J-774 (311). Instead, human macrophage like cell-line U-937 (ATCC 1593.2 CRL) (148, 290) was used. Suspension cultures of U-937 cells were grown in RPMI 1640-10% heat-inactivated fetal calf serum (FCS) (Atlanta Biologicals, Norcross, GA) medium in 75-cm² tissue culture flasks for 3 d before overnight treatment with 0.4 µg/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma) to transform U-937 into an adherent state. PMA-containing supernatants were removed the next day, and the adherent cells were washed with Hanks' Ca²⁺- and Mg²⁺-free balanced salt solution (HBSS). Adherent U-937 cells were released by gentle rocking with 3-mm-diameter glass beads. Released cells were recovered by centrifugation, washed in HBSS, quantitated with a hemocytometer, and diluted in RPMI 1640-10% FCS medium to a density of 2×10^5 cells per ml. U-937 cells (1 ml/well) were plated into each well of a 24-well tissue culture plate (Costar) and incubated overnight under 5% CO₂ at 37°C. Cultures of *M. smegmatis* 1-2c containing the *M. tuberculosis* plasmid library were grown on 7H10 agar plates with 50 µg of hygromycin B per ml for 3 to 4 d. Inocula were prepared by swabbing the plate growth into HBSS, sedimenting the mycobacteria at $1,300 \times g$ for 10 min, resuspending in HBSS, vortexing for 30 s, and centrifuging again at $250 \times g$ for 5 min. This yielded a

supernatant consisting almost entirely of single mycobacterial cells, as observed by phase-contrast microscopy. On the assumption that an optical density at 650 nm (OD_{650}) of 0.1 equals 10^8 CFU per ml, the inoculum was diluted to a density of 2×10^6 CFU per ml in RPMI 1640-10% FCS containing 2.5% fresh human serum (Omega Scientific). U-937 cells resemble human mononuclear phagocytes in that phagocytosis of mycobacteria is greatly augmented in the presence of fresh human serum (complement component C3) (271). Aliquots of the inocula were routinely plated on 7H10 plates containing hygromycin B for CFU determinations to confirm that equivalent bacterial inocula were used in all experiments. After 20 min of opsonization at room temperature, 1 ml of inoculum was added to each well containing 2×10^5 U-937 cells to give a multiplicity of infection (MOI) of 10. At this MOI, no cytotoxicity was observed. The 24-well plates were incubated for 2 h at 37°C in a 5% CO₂ incubator. Acid-fast staining showed that after 2 h of incubation approximately half of the U-937 cells contained at least one *M. smegmatis* organism. The infected monolayers were then washed once with warm HBSS and treated with RPMI 1640-10% FCS containing 200 µg of amikacin per ml for 1 h at 37°C to kill extracellular organisms. The monolayers were washed again with HBSS, and those monolayers in which survival for 24 and 48 h was to be measured were incubated in medium containing 20 µg of amikacin/ml to prevent extracellular growth of any bacteria that might be released by premature lysis of infected U-937 cells. Cells in duplicate wells were lysed at 3, 24, and 48 h post-infection by adding 1 ml of water, waiting for 30 min, and vigorously pipetting five times to ensure cell lysis and the release of surviving

intracellular bacteria. The lysates were serially diluted in 7H9 broth and plated onto 7H10 agar plates containing hygromycin B. CFUs were counted after incubation at 37°C for 3 d. *M. smegmatis* (pOLYG) was included in every assay as a negative control.

Clones that were recovered on plates at 24 and 48 h in each passage were pooled and passed through the U-937 survival assay for the next passage. A total of 6 passages were carried out to enrich for recombinant *M. smegmatis* with increased capacity for intracellular survival. After each step in the passage, bacterial pools were frozen at 70 °C for further analysis.

The intracellular survival assay for *M. tuberculosis*

The *M. tuberculosis* inocula and the macrophage monolayers were prepared as described previously. *M. tuberculosis* strains were cultured in 50 ml 7H9-ADC-Tween 80 with appropriate antibiotics (hygromycin B, 50 µg/ml and/or kanamycin, 25 µg/ml) at 37 °C for two weeks. Single cell suspensions were obtained by first vortexing the bacteria with glass beads (2 mm) and then centrifuging the mixture at 1,000 rpm for 5 min with brake off (setting 8) in a Beckman Model T J-6R centrifuge. The concentration of bacteria was adjusted accordingly. Monolayers were infected with mycobacteria at a MOI of either 1 or 0.1. After 4-h infection, the extracellular bacteria were removed, the monolayer was washed three times with warm RPMI 1640 containing 1% human serum (R1), and fresh RPMI 1640 with 10 % human serum (R10) was added and cultures incubated at 37 °C in 5% CO₂ for 6 additional days.

Fresh R10 was added at day 3. Antibiotics were not used at any point. Controls for extracellular growth of *M. tuberculosis* were obtained by inoculating 10^5 CFU/ml in 1 ml of R10 containing a sonicated lysate of 2×10^6 U-937 macrophages which had been passed through a 0.22 μ m-pore-size filter to remove cell debris. The extracellular growth of *M. tuberculosis* in R10 culture medium alone did not occur. The numbers of CFUs were determined at 4 h, and at 2, 4, and 7 d after infection. Because the lysis of infected macrophages resulted in extensive loss of adherent cells, the number of intracellular bacteria at each time point was determined by using total lysates. Briefly, 0.1 ml of 1 % Triton X100 was added directly into the culture medium to lyse macrophages for 30 min, the total lysates were then serially diluted in 7H9 plus ADC and plated in duplicate on 7H10 plus OADC. CFUs were counted after incubation at 37 °C for 2 to 3 weeks.

Transmission electron microscopy

U-937 cell monolayers were prepared in 24-well tissue culture plates as described for the intracellular survival assay and infected with *M. smegmatis*(p69) at an MOI of 50. This high MOI was used to assist in the observation of *M. smegmatis*(p69) in the transmission electron microscopy preparations. At 3 and 24 h after infection, monolayers were washed and fixed for 1 h at room temperature in 0.1 M phosphate buffer (pH 7.2) containing 2 % glutaraldehyde. Cells from fixed monolayers were then released into suspension by using a rubber policeman. Specimens were postfixed in 2 % osmium tetroxide, dehydrated via graded alcohol steps, and embedded in Spurr low-

viscosity resin. Sections were cut, stained with uranyl acetate and lead citrate, and viewed on a Philips CM12 transmission electron microscope. We thank Peggy McClusky of Arizona Research Laboratories Division of Biotechnology Imaging facilities, University of Arizona for expert technical assistance with the electron microscopy studies.

Production of affinity-purified antibody against Eis

Affinity-purified rabbit antibodies against the Eis protein were produced using synthetic peptides for this study by Quality Controlled Biochemicals (QCB), Inc., Hokinton, MA. One potentially immunogenic peptide sequence was selected from the deduced Eis amino acid sequence, a 18-mer (CAANRLRTKDSQLLRR LD) region from the carboxy terminus of the Eis protein. The peptide was synthesized and conjugated to the carrier protein KLH by a cross linker and used to immunize rabbits via subcutaneous injection mixed in Complete Freund's Adjuvant. Rabbits were boosted at least five times and test bleeds were taken at each stage to monitor antibody titers against Eis peptides via ELISA. ELISA were performed using peptide bound to BSA. ELISA results demonstrated the production of high titer antibodies against the Eis peptide. Antibodies generated against Eis peptide were then affinity-purified. In brief, rabbit serum was passed over an agrose-based column thio-coupled to the peptides. The column was washed, the antibody eluted using a low pH glycine buffer, and recovered affinity-purified antibodies were dialyzed in PBS. The affinity-purified anti-Eis antibody was stored frozen until use in Western blots diluted 1:1250.

Western blot analysis

Western blot analysis was performed as previously described (1). Lysates of *M. smegmatis* samples were prepared from 3 d cultures grown in 50 ml of 7H9 broth. Lysates of *M. tuberculosis* H37Ra and H37Rv samples were prepared from 14 d cultures grown in 50 ml of 7H9-ADC-Tween medium. Before use, lysates of *M. tuberculosis* H37Rv samples were sterilized using a 0.22- μ m filter. Cultures were centrifuged in 15-ml screw-cap conical tube for 10 minutes at 3,000 rpm, and pellets were washed once with phosphate buffered saline (PBS) to avoid contaminating proteins from the culture medium. Cell pellets were then transferred to microcentrifuge tubes and resuspended in 1.2 ml ice-cold PBS. One hundred microliters of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) were added and mixtures were vortexed at 4 °C for 30-60 min using a Vortex-Genie Turbomix device (Fisher Scientific). The cell suspension was centrifuged at 12,000 rpm for 1 min and the supernatants were collected. Protein concentrations were then determined using the BCA colorimetric assay (Pierce, Rockford, IL), with bovine serum albumin (BSA) as a standard, following the manufacturer's directions. The cell-free lysates corresponding to approximately 50 μ g of protein were mixed with one-tenth volume 10 % sodium dodecyl sulfate (SDS), and boiled for 10 minutes before samples were loaded. Samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for 4-5 h at 100 V and 70 mA. The proteins were then electroblotted onto a Trans-Blot Transfer Nitrocellulose membrane (Bio-Rad, Hercules, CA) using an electroblotting apparatus (Bio-Rad) at 4 °C overnight (300). The next day, the

membrane was blocked with 10 % non-fat milk in Tris-buffer (20 mM Tris (pH7.5), 0.5M NaCl buffer containing 0.1% Tween 20) for 1 h, and reacted with affinity-purified anti-Eis antibody (0.5 µg/ml) for 4 h. The membranes were then washed and reacted with secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG) (Zymed, San Francisco, CA) overnight at 4 °C. Bands were detected using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (BCIP) (Boehringer Mannheim) as substrate (263). Band intensity was quantitated using the AlphaImage 2000 Documentation and Analysis System software using the spot densitometry function (AlphaInnotech, San Leandro, CA).

Nucleotide sequence accession number.

The nucleotide sequence of the *eis* gene has been submitted to GenBank database and assigned the accession no. AF144099.

Statistical analysis of data.

Results of assays for intracellular survival are expressed as means \pm standard deviations from at least three independent experiments done in duplicate and performed on different days. Differences between various groups were assessed by using the Student's *t* test. The level of significance was set at 0.001.

Table 1. Plasmids used in this study.

Plasmids	Relevant characteristics	Reference or Source
pUC18	ColE1 cloning vector, Amp ^r	New England Biolabs
pSP72	<i>E. coli</i> cloning vector, Amp ^r	Promega
pGEM-7Zf(+)	<i>E. coli</i> cloning vector, Amp ^r	Promega
pOLYG	<i>E. coli</i> -mycobacterial shuttle vector, Hyg ^r	(133)
pMV306	Mycobacterial integrating vector, integrates into the <i>attB</i> site, Kan ^r	(287)
pRL498	pUC-based vector, containing 1.3-kb hygromycin resistance cassette, Hyg ^r , Kan ^r	C. Barry III ^a
pMJ10	<i>ts oriM</i> ; <i>sacB</i> counterselection, Kan ^r Gent ^r	(237)
p69	pOLYG with 2.99-kb <i>M. tuberculosis</i> genomic DNA fragment containing the <i>eis</i> gene	This study
p62-8	pSP72 with 1.3-kb <i>Bam</i> HI- <i>Sph</i> I fragment	This study
p6216	pSP72 with 1,7-kb <i>Sph</i> I- <i>Bam</i> HI fragment	This study
p69-8	pOLYG with 1.3-kb <i>Bam</i> HI- <i>Hind</i> III fragment from p62-8	This study
p69-16	pOLYG with 1.7-kb <i>Bam</i> HI- <i>Hind</i> III fragment from p62-16	This study
p62-97	pSP72 with 1.6-kb <i>Apo</i> I- <i>Pvu</i> I <i>eis</i> -containing fragment from pOLYG	This study
p69-96	pOLYG with 1.6-kb <i>Cla</i> I- <i>Hind</i> III fragment from p62-97	This study
p69-97	pOLYG with 1.6-kb <i>Eco</i> Rv- <i>Hind</i> III fragment from p62-97	This study
p69-96A	p62-96 lacking the 367-bp <i>Apa</i> I fragment	This study

Table 1. (continued)

Plasmids	Relevant characteristics	Reference or Source
p6301	pMV306 with 1.6-kb <i>ClaI-HindIII</i> fragment from p62-97	This study
p62-37	1.3-kb hygromycin gene released by <i>XbaI</i> from pRL498 replacing an 367-bp internal <i>ApaI</i> fragment from p62-97	This study
p62-39	pGEM-7Zf(+) with 2.5-kb <i>ClaI-HindIII</i> fragment from p62-37	This study
p6308	pMJ10 with 2.5-kb <i>XbaI-BamHI</i> fragment from p6239	This study

^a National Institutes of Health.

Table 2. Bacterial strains used in this study.

Strains	Description	Reference or source
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (Δ80lacZ ΔM15)</i> <i>hsdR17recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco BRL
HB101	<i>Δ(gpt-proA)62 leuB1 glnV44 ara-14</i> <i>lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13</i>	Gibco BRL
<i>M. smegmatis</i> 1-2c	derivative of strain mc ² 6, high transformation efficiency	(332)
<i>M. tuberculosis</i>		
H37Ra	attenuated H37Rv	D. Young ^a
Ra610	double-crossover recombinant of H37Ra with p6308, Δ <i>eis::hyg</i> , Hgr ^r	This study
Ra610-16	Ra610 containing integrated copy of p6308 Δ <i>eis::hyg</i> Hgr ^r , Kan ^r	This study
H37Rv	Virulent laboratory strain (ATCC 27294)	CSU ^b
Rv6	double-crossover recombinant of H37Rv with p6308, Δ <i>eis::hyg</i> , Hgr ^r	This study
Rv8-1	Rv6 containing integrated copy of p6308 Δ <i>eis::hyg</i> , Hgr ^r , Kan ^r	This study

^a Imperial College School of Medicine at St. Mary's, London, England^b Colorado State University.

Table 3. Primers used in this study.

Primers	Sequence
pOLYG For	5'-TCGAGGTCGACGGTATCG- 3'
pOLYG Rev	5'-GATTACCAGATCTGGCTCG- 3'
69.2f	5' -CAGGCGATGAGCTGGATA- 3'
69.2r	5' -GCTCCGGGTCATAGTGTG- 3'
69.3f	5' -CAGACACTGTCGTCGTA- 3'
69.3r	5' -GGTGCGCTCCAAGGTA- 3'
5' <i>eis</i> -probe 1 (69.4f)	5'-GGATCCGTCAGACCCACCGAGCAT-3'
3' <i>eis</i> -probe 1 (69.8r)	5'-CGGATCCCCATCCATGGCGTGT-3'
5' <i>eis</i> -probe 2 (69.91f)	5'- ATCGATCGGGTTTGCCAGCTTGT-3'
3' <i>eis</i> -probe 2 (<i>eis</i> -dig-r)	5' -GCGTCGGCGCCACCGCGACG-3'

RESULTS

Screening of *M. smegmatis* transformed with *M. tuberculosis* H37Rv DNA for enhanced survival in U-937 cells.

To enrich for recombinants with increased ability for intracellular survival, *M. smegmatis* transformants containing the *M. tuberculosis* H37Rv DNA plasmid library were used to infect monolayers of U-937 (148) as described in Materials and Methods. The *M. smegmatis* transformants containing the *M. tuberculosis* H37Rv DNA plasmid library were passed through the U-937 for a total of six passages to enrich for recombinant *M. smegmatis* with increased ability for intracellular survival. After each step in the passage, clones that were recovered on plates from the 24 or 48 h time points were pooled and frozen at -70 °C for further analysis.

The shuttle vector pOLYG is a multicopy, hygromycin-resistant plasmid (133). Since hygromycin B was not present during serial passages and assays for survival in U-937 cells, it was necessary to verify that antibiotic selection pressure is not required to maintain the plasmid library in *M. smegmatis* within the macrophage monolayers. When infected monolayer lysates from different passages were plated on 7H10 agar plates with or without hygromycin B, equivalent numbers of *M. smegmatis* CFU were recovered (data not shown). This demonstrates that hygromycin B is not required for maintenance of the genomic plasmid library in *M. smegmatis* during passage in the U-937 cells. After the third passage, ten clones were randomly selected for isolation of their plasmids to verify that insert DNA was present. At least 80% of isolated plasmids were found to containing an average insert DNA of 5.4-kb (data not shown).

Isolation of clone 69 (p69), a *M. smegmatis* transformant with increased resistance to killing by U-937 cells.

After the third passage in U-937 cells, one hundred twenty independent clones were randomly selected and screened individually in the intracellular survival assay to identify individual *M. smegmatis* recombinants with enhanced survival as compared to *M. smegmatis* containing only the vector pOLYG. Twenty-one of the recombinants showed 2-fold or greater increased survival 48 h after infection. One clone (p69) demonstrated significantly enhanced survival, ranging from 2.4- to 5.3-fold at 24 and 48 h after infection, respectively (Figure 1), and this clone was further characterized.

To verify that the enhanced intracellular survival phenotype was due to the *M. tuberculosis* DNA insert and not to a chromosomal mutation which might have occurred in *M. smegmatis* during passage in U-937 cells, the p69 recombinant plasmid DNA was isolated and retransformed into non-passaged *M. smegmatis* cells. The retransformed p69 showed identical levels of survival in U-937 cells comparable to that of the original p69 transformant. All data presented in this dissertation were obtained using p69 plasmids or derivatives retransformed into non-passaged *M. smegmatis*.

In the survival assay, amikacin (200 µg/ml) is used initially to kill uningested extracellular *M. smegmatis*. Following this 1 h treatment, 20 µg of amikacin/ml is present to prevent bacterial replication in the tissue culture medium. Theoretically, it might be possible to select for amikacin-resistant mutants in the survival assay. To clarify this, the minimal inhibitory concentrations (MICs) of amikacin were determined for *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) recovered after passage in the

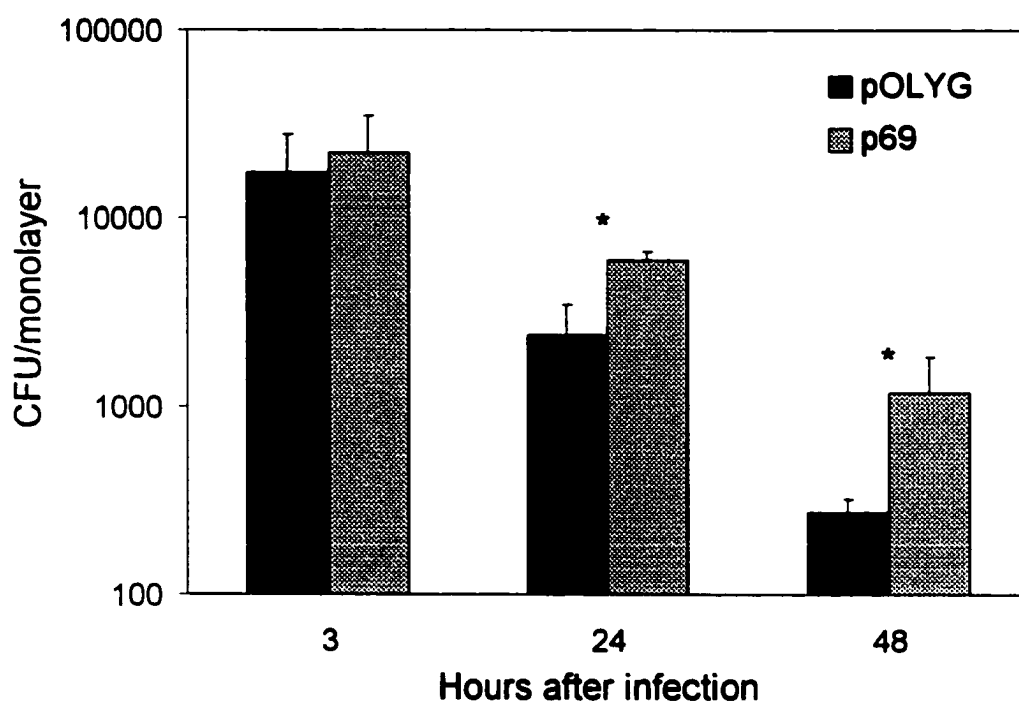


Figure 1. Survival of *M. smegmatis* containing pOLYG or p69 in U-937 cells. Surviving intracellular bacteria were counted at 3, 24, and 48 h after infection. The data represent the mean \pm standard deviations from 3 independent experiments performed on different days. *, $p < 0.001$ as compared to pOLYG at each time point.

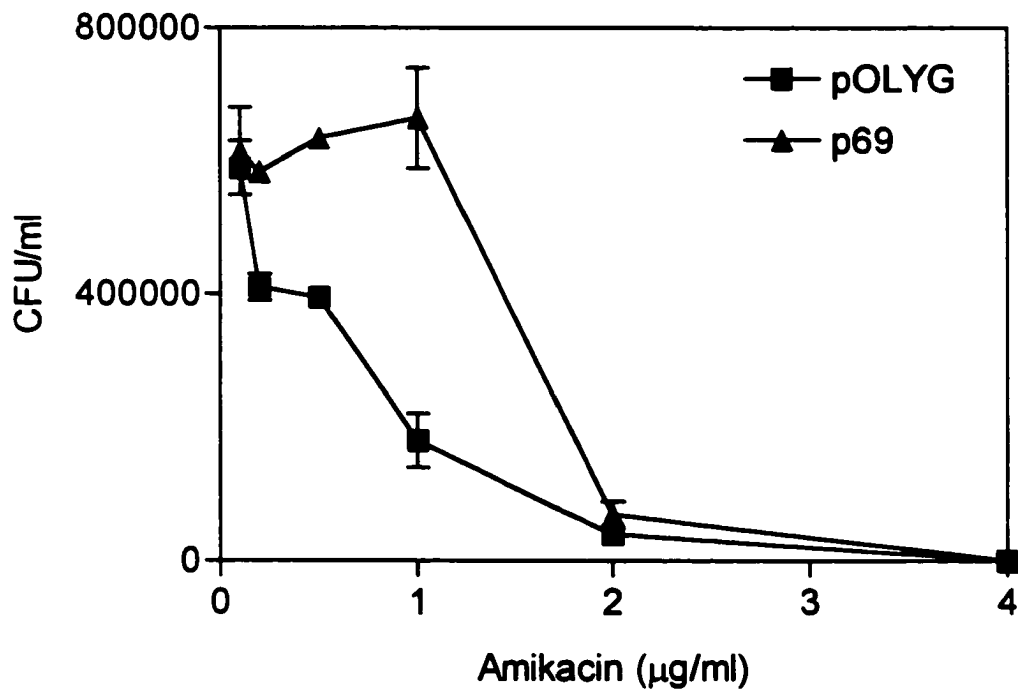


Figure 2. Resistance of *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) to amikacin. Dilutions of each clone ($\sim 6 \times 10^5$ cells) were plated on 7H10 plates containing various concentrations of amikacin. CFUs were counted after 3 d incubation at 37 °C. Data presented are from a representative experiment, each concentration was done in duplicate. Results are plotted as means \pm standard deviations.

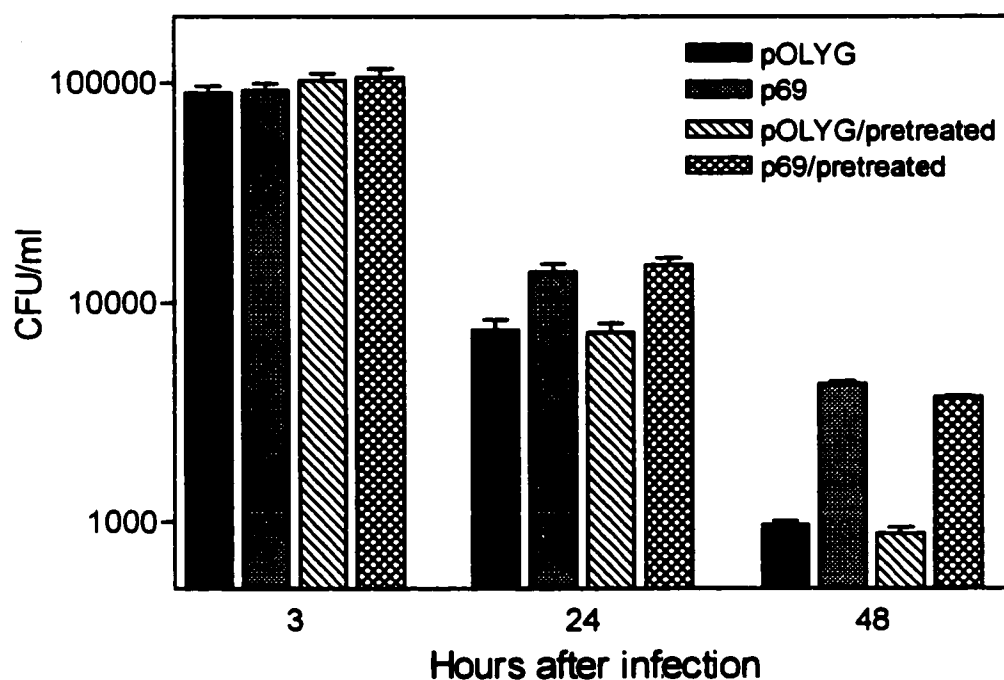


Figure 3. Effect of amikacin on the intracellular survival of *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) within U-937 cells. U-937 monolayers were pretreated with amikacin at 20 $\mu\text{g/ml}$ for 48 h and then at 200 $\mu\text{g/ml}$ for 1 h prior to infection. Normally prepared U-937 monolayers were used as a control. An MOI of 10 was used in the study. Surviving intracellular bacteria were counted at 3, 24, 48 h after infection. Results are presented as the means \pm standard deviation from two independent experiments done in duplicate.

survival assay and for unpassed *M. smegmatis* transformed with each vector. In both passaged and freshly transformed *M. smegmatis*(pOLYG) and *M. smegmatis*(p69), the MIC was 4 µg/ml (Figure 2). Thus, *M. smegmatis*(p69) has no increased resistance to amikacin.

We also sought evidence that amikacin kills extracellular microbes only and does not inhibit the intracellular survival of *M. smegmatis*. If the levels of amikacin used in the survival assay do indeed kill ingested *M. smegmatis*, then doubling the exposure of U-937 cells to the antibiotic should reduce intracellular survival. U-937 cells were pretreated with amikacin at 20 µg/ml for 48 h and then at 200 µg/ml for 1 h prior to infection with *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) in the survival assay. A comparable population of U-937 cells not pretreated with the antibiotic was also infected with the same inocula, and the survival of the four sets of *M. smegmatis*-infected U-937 cells were then compared in the customary assay (Figure 3). Equivalent numbers of *M. smegmatis* transformants were recovered in both the amikacin-pretreated (two rounds of amikacin exposure) and non-pretreated (one round of amikacin exposure) groups at 3, 24, and 48 h after infection. Therefore, amikacin is not significantly internalized into U-937 cells to levels that interfere with the intracellular survival of *M. smegmatis*.

Survival of *M. smegmatis*(p69) in primary human monocytes.

The survival of *M. smegmatis*(p69) in human monocyte-derived macrophages was also investigated. Human monocytes were infected with *M. smegmatis*(pOLYG) and *M. smegmatis*(p69) as described for U-937 cells, except 15 % autologous human serum and

5 µg/ml amikacin were used to maintain the monocytes during the infection. The results of infection assays are presented in Figure 4. Although Barker *et al.* reported that adherent human monocytes do not kill *M. smegmatis* (16), Plum *et al.* subsequently found that the bacilli are killed by these cells if the MOI is low (244). Our result confirmed that *M. smegmatis* was indeed killed by human monocytes and at about the same rate as in U-937 cells. We observed that, just as in U-937 cells, *M. smegmatis*(p69) survives better than its vector control *M. smegmatis*(pOLYG). *M. smegmatis*(p69) showed enhanced survival, ranging from 3.9- to 9.5- fold at 24 and 48 h after infection, respectively. These results indicate that *M. smegmatis*(p69), which showed enhanced survival in the U-937 intracellular survival assay, does indeed demonstrate comparable enhanced survival behavior in a human macrophage system. These results confirm the validity of using U-937 cells for the initial selection of library clones with enhanced intracellular survival.

Electron microscopy.

Electron microscopy was performed to confirm that *M. smegmatis*(p69) had indeed been internalized by U-937 cells and was present intracellularly. Figure 5 shows typical observations of intracellular *M. smegmatis*(p69) in the cytoplasm of U-937 cells. At 3 h postinfection, numerous *M. smegmatis* bacilli were present in the cytoplasm of U-937 cells either in tightly fitting phagosomes (Figure 5A) or in more spacious phagosomes (Figure 5B). The mycobacteria were also seen within phagosomes and not free in the cytoplasm. Similar electron micrographs were obtained at 24 h after infection (data not shown). These electron micrographs of *M. smegmatis* in U-937 cells are comparable to

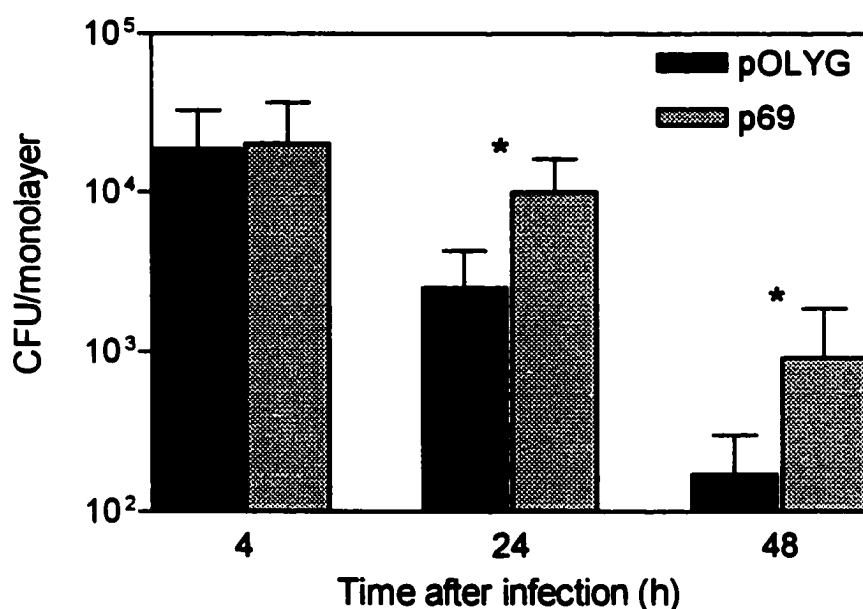


Figure 4. Survival of *M. smegmatis* containing pOLYG or p69 in human monocytes. Monocyte monolayers were infected at an MOI of 10 for 2 h. The fresh RPMI medium containing 200 µg/ml amikacin was added to kill the extracellular mycobacteria. After 1 h killing, medium was changed to RPMI-15 % autologous serum containing 5 µg/ml amikacin. The numbers of CFU were determined at 3, 24 and 48 h after infection. The data represent the mean ± standard deviations from 3 independent experiments using the blood from 3 PPD-negative healthy young adults on different days. *, $p < 0.001$ as compared to pOLYG at each time point.

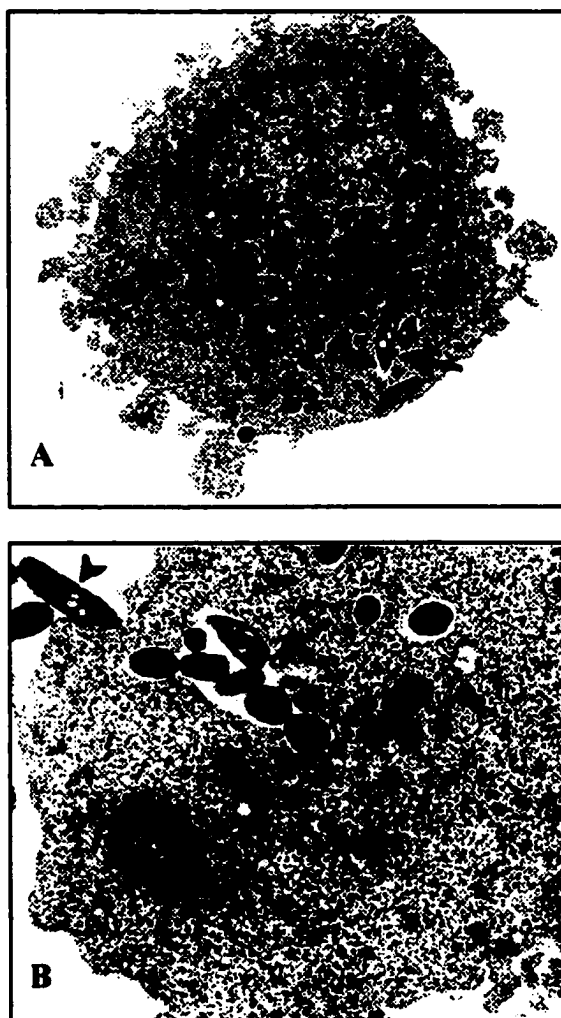


Figure 5. Electron micrographs demonstrating intracellular *M. smegmatis*(p69) within U-937 cells at 3 h after infection. (A) Several *M. smegmatis*(p69) bacilli (arrows) are present in tight-fitting phagosomes in the cytoplasm of the macrophage-like cell. Magnification, X 8,500. (B) Higher magnification of an area of another U-937 cell which contains *M. smegmatis*(p69) in more spacious phagosome (arrows). Note bacilli probably in the early stages of internalization (arrow head). Magnification, X 13,600.

those of *M. smegmatis* in human mononuclear phagocytes (16).

Restriction digestion and sequence analysis of p69.

To facilitate analysis of p69, the plasmid was directly transformed by electroporation from *M. smegmatis* into *E. coli* DH5 α cells. Restriction mapping indicated that p69 contained a 2.99-kb DNA insert. The p69 insert was sequenced and analyzed. The nucleotide sequence is shown in Figure 6. The insert DNA was GC rich (68% G+C) and was identical to a nucleotide sequence found in the *M. tuberculosis* genome database (66). This is a 9-kb region in which none of the potential ORFs has a known function (Figure 7). Nucleotide sequencing revealed the presence of three potential ORFs designated ORF1, ORF2, and ORF3 in the insert (Figure 8). These ORFs correspond to the hypothetical genes Rv2415c, Rv2416c, and Rv2417c, respectively, in the *M. tuberculosis* genome (66).

The deduced amino acid sequences of the three ORFs were used in searches of the GenBank/EMBL and SWISSPROT databases with the BLAST and FASTA programs. We also utilized a recently described position-specific iterated BLAST (PSI-BLAST) (3), a new sequence homology searching program which can identify highly significant pattern homologies in proteins where little primary sequence homology exists, to search for homologues of the 3 potential ORFs (<http://www.ncbi.nlm.nih.gov/blast/psiblast.cgi>). Unfortunately, no homologues of known function were found for these ORFs. ORF1

Figure 6. Nucleotide sequence of *eis* and the surrounding open reading frames (ORFs). Single letter amino acid abbreviations are shown below the nucleotide sequence. Relevant restriction endonuclease recognition sites are indicated above the sequence. Arrows indicate direction of transcription for each of the ORFs. Asterisks indicate translation stop codons. Putative ribosome binding site (RBS) and putative –35 and –10 promoter sequences are underlined. Three potential start codons for the Eis protein are boxed. The start codon based upon N-terminal sequence analysis is boxed in bold. The amino acids translated differently from the first two start codons are in shade.

ggccgacctgcgcgaacagtggtcgatccgccagggtcccgtgcatatct 50
 tgcttgacggcctcgacctgcgcgacgggtgtggacgaaatccccgatgac 100
 atccacaagcgccacgccaccaccgctggggcgacccccggttgagctgtc 150
 cgccgcctaccaacgggcgttggcggacagtggcggcgacggggtagtg 200
 cggtgcacatttcgtcggcgctgtcgggtacctttcgagccgccgagctg 250
 accgcggcggaactaggtcccgccttaggggtgatcgactcgaggtcggc 300
 Rv2417c →
 cgcgatgggcgtcggtttcgcggcactggcggccgggcgggcagccgccg 350
 M G V G F A A L A A G R A A A A
 caggcgatgagctggatacggtcgcgcgcgcagcggctgcggcggttaagc 400
 G D E L D T V A R A A A A A V S
 cggattcacgcgttcgtcgtctgtagcgcgggttgacaaatctgcgccgcag 450
 R I H A F V A V A R L D N L R R S
 cgggcgcacatcagtggggccaaggcatggttgggcaccgcgctggcgctca 500
 G R I S G A K A W L G T A L A L K
 agccgctgctgtcagtcgacgacggaaaacttggttctggtccaacggggt 550
 P L L S V D D G K L V L V Q R V
 cgcaactgtgagcaacgcgacggcggtgatgatcgaccggggtttgccagct 600
 R T V S N A T A V M I D R V C Q L
 tgtcggcgaccgccccgccgctctcgcgggtgcatcacgtcgccgaccg 650
 V G D R P A A L A V H H V A D P A

PvulI

cagctg cgaacg acgtgg cggcggc gctggc ggagcg gctgcc ggcgct gt 700
A A N D V A A A L A E R L P A C

gagccggccatggtgaccgccatgggaccgggtacttgctctgcacgtcgg 750
E P A M V T A M G P V L A L H V G

tgccggagccgtcggggtatgcgctcgacgtgggagcgctcgccgccagcgt 800
A G A V G V C V D V G A S P P A *

ApoI

aacgtcacggcgaatttcgtcactgattctcgcagtggcgtcacactggc 850

-35
-10
900
 ggggctacccgcatcgcgatcctttgccagacactgtcgtcgtaatat

tcacgtgcacgtggccgcgcatatgccacagtcggattctgtgactgtg 950
 NdeI RBS eis →
 [] [] []
 N P Q S D S V T V

accctgtgtagcccgaccgaggacgactggccgggggatgttcctactggc 1000
T L C S P T E D D W P G M F L L A

cgcgggccagtttcaccgatttcacggccctgaatcagcgaccgcctggc 1050
A A S F T D F I G P E S A T A W R

ggaccctggtgccaccgacggagcgggtggtggtccgcgatggtgccggc 1100
T L V P T D G A V V V R D G A G

ccgggttctgaggtggtcgggatggcgctgtacatggatctgcggttgac 1150
P G S E V V G M A L Y M D L R L T

ggtgcctggtgaagtggtgctcccgaccgccggtctcagtttcgtcgcg 1200
V P G E V V L P T A G L S F V A V

tggcgccgacgcacgcgcggcgcggttgctgcgcgcgatgtgcgccgaa 1250
A P T H R R R G L L R A M C A E

SphI

ctgcaccgcccgcatagccgattccggctatccgggtcgcggcactgcatgc 1300
 L H R R I A D S G Y P V A A L H A

NheI **ApaI**

tagcgagggcggcacatctacggccgggttcgggtacgggcccgcgtaccacct 1350
 S E G G I Y G R F G Y G P A T T L

tgcattgagctgacgggtcgaccgacgcttcgcgcgctttcacgccgacgca 1400
 H E L T V D R R F A R F H A D A

ccgggcgggcggcctaggtggcagcagcgtccgggttggtcagaccaccca 1450
 P G G G L G G S S V R L V R P T E

gcatcgcgggcgagtttgaggcgatctacgagcgatggcgccagcaggtgc 1500
 H R G E F E A I Y E R W R Q Q V P

cgggcgggctgctacgcccgcaggtgctctgggacgagctgctggcagaa 1550
 G G L L R P Q V L W D E L L A E

tgcaaagccgcgcccgggtggagaccgtgaatcggttcgcgttactgcatcc 1600
 C K A A P G G D R E S F A L L H P

NheI

cgacgggtacgcgctgtaccgggtggatcgacccgatctcaagctagcgc 1650
 D G Y A L Y R V D R T D L K L A R

gcgtcagcgaactcagggcggttaaccgcagatgcgcattgtgcgttgtgg 1700
 V S E L R A V T A D A H C A L W

ApaI

cgggccctgattggcctcgactccatggagcgaatcagcatcatcaccca 1750
 R A L I G L D S M E R I S I I T H

tccacaggaccggttacccacctgctcaccgatacccgactggcccgcga 1800
 P Q D P L P H L L T D T R L A R T

ctacctggcgccaggacggcctgtggttgcgcatcatgaacgtaccggcc 1850
T W R Q D G L W L R I M N V P A

XhoI

gcactcgaggcgcggttggttacgctcacgaagttggcgagttttccacggt 1900
A L E A R G Y A H E V G E F S T V

cctcgagggtatccgatggcgggccggttcgcgctcaagatcggtgacggcc 1950
L E V S D G G R F A L K I G D G R

gtgcgcggtgtacccccgaccgatgcggcagccgagatcgaaatggatcgg 2000
A R C T P T D A A A E I E M D R

gacgtactgggcagcctttaccttgagcgccacgcgcttcgacgcttagc 2050
D V L G S L Y L G A H R A S T L A

PvuII

cgccgctaaccggttgcgcaccaaagattcccagctgcttcgctcgactcg 2100
A A N R L R T K D S Q L L R R L D

acgcggcggtttgccagtgatgttcccgtccagaccgcgcttcgagttctga 2150
A A F A S D V P V Q T A F E F *

aggccgtgctagggccggcgctaggctgacgggcttttcggcggtggtcagc 2200

Rv2415c →

gacccgcgtgctgcgcgccggcttcggtcgccacacgccatggatgggga 2250
M D G D

PvuI

tcggccgcgcggctcagcactcgcggttcggttgccgagtacactctcg 2300
R P R G S A L A D R V A E Y T L D

atcgcggtgagccgagtcgggatggacgtcgcagcccgggtgctggttgtt 2350
R G E P S P D G R R S P V L V V

cggggatgttggcgcggtttgtcccatcttgatccatcgccaaagcggct 2400
R G C W R G L S H L D P S P K R L

tgtccacagcctcggattgatccacagcagggcagcgcgacgccgtcgtg 2450
V H S L G L I H S R A A R R R R A

PvuII

cgcgccggttcttggcgggtccccgcagctgcggtcggcccatgcgaacaga 2500
R R F W R S P Q L R S A H A N R

EcoRv

actgcccgccgagcgcactgcaaaggcggctcggtgccgtcccggatatcg 2550
T A R R A T A K A A R C R P G Y R

actcgcacgccgcatctgcacacttagaccggagccacacgatccaacg 2600
L A R R I C T L R P G A T R S N G

gacgacgggtccagaccacgacgagccacgcgacgatccgaactcgctgct 2650
R R S R P R R A T R R S E L A A

gccgcgctggcttccccgacacttcccgtgggcaagggtgggcggacagga 2700
A A L A S R H F P W A R L G G Q D

tacgcgcagatccgggccgtgccggcgccgtcgcatcggcggtgatcgcc 2750
T R R S G P C R R R R I G G D R R

gccctcgcggtgctggtgacggtattcaccttgatccgcgaccggactga 2800
P R G A G D G I H L D P R P D *

gccggtaatgtcagccaaacttcctccggtagagccggtttcgccgacga 2850

accctaggtcgtcggcaagcccgggctcgccggaccgttccggcctgccg 2900

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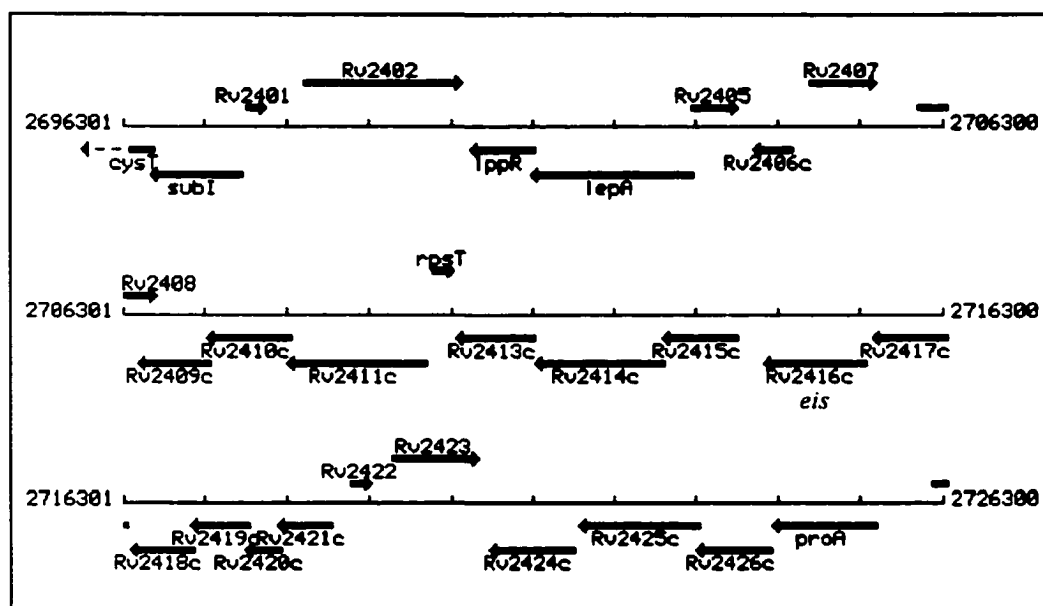


Figure 7. Open reading frames (ORFs) or genes adjacent to *eis* (2416c) on the 2696.3-kb to 2726.3-kb region of the *M. tuberculosis* genome. Arrows mark the position and direction of transcription. ORFs above the line are transcribed using the coding strand. ORFs below the line are transcribed using the complement strand (c). *cysT*, Cys tRNA gene; *subI*, (thio)sulphate binding protein gene; *lppR*, a probable lipoprotein gene; *lepA*, GTP-binding protein *lepA* gene; *rpsT*, 30s ribosomal protein S20 gene; *proA*, gamma-glutamyl phosphate reductase gene.

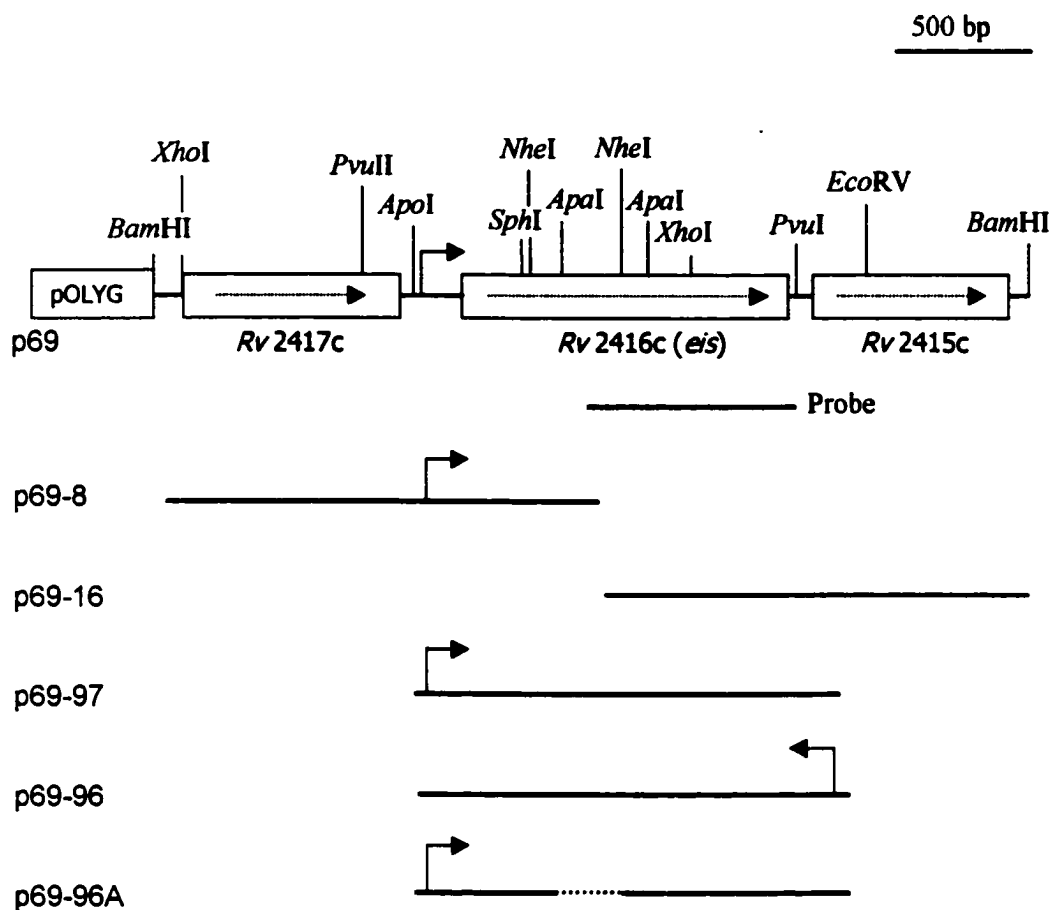


Figure 8. Restriction map of p69 and its deletion derivatives. The boxes indicate the 3 ORFs revealed by sequencing. The dashed arrows mark the direction of transcription for each of the ORFs. The putative promoter is shown by an arrowhead. The dotted line indicates an in-frame deletion. Relevant restriction endonuclease recognition sites discussed in the text are indicated. The internal *eis*-1 probe used for Southern hybridization is depicted.

(2417c) is predicted to encode a 280-amino acid conserved hypothetical protein which has some similarity (31% identity, 45% similarity) to the hypothetical protein SCC123.07c of *Streptomyces coelicolor* (250). ORF2 (2416c) potentially encodes a 44-kDa conserved hypothetical protein (404 a.a.). Some homology (34% identity) was found between ORF2 and a hypothetical 45-kDa protein (*orf5*), of unknown function, downstream from the *amfC* gene of *Streptomyces griseus*, which is involved in aerial mycelium formation in this microbe (174). ORF3 (2415c) potentially encodes a 297-amino acid conserved hypothetical protein, which is similar to a putative DNA-binding protein of *Streptomyces coelicolor* (identity 38%, similarity 50%) (250).

Moreover, the DNA sequence analysis reveals a putative promoter region and a ribosome binding site (RBS) upstream of ORF2 (Figure 7). This suggests that ORF2 of p69 is likely to be the gene which confers the enhanced intracellular survival phenotype on *M. smegmatis* (311).

Subcloning and deletion analysis of ORF2.

Deletion analysis of the p69 insert DNA was carried out to verify that intact ORF2 is indeed essential for the enhanced intracellular survival phenotype observed in *M. smegmatis*(p69) (Figure 8). Plasmid p69-8 contains a complete ORF1 and the 5' half of ORF2, while plasmid p69-16 contains the 3' half of ORF2 and an intact ORF3. Both plasmids p69-96 and p69-97 contain a 1.6-kb *ApoI-PvuI* fragment with the intact ORF2 and its putative promoter region, but in opposite orientations. Plasmid p69-96A is an in-frame deletion of p69-96, in which ORF2 has been disrupted by removal of the 367-bp

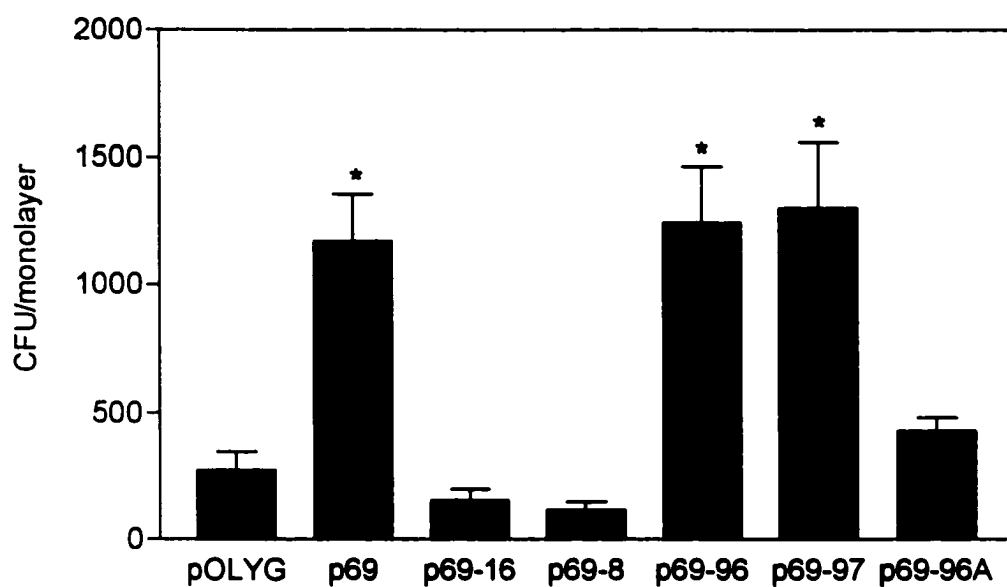


Figure 9. Survival of *M. smegmatis* containing pOLYG, p69, and deletion derivatives of p69 in U-937 cells. Surviving intracellular bacteria were counted 3, 24, 48 h after infection, but only the 48 h values are presented. The data represent the mean \pm standard deviations from three independent experiments performed on different days. *, $p < 0.001$, as compared to pOLYG.

ApaI fragment. These deletion constructs were electroporated into *M. smegmatis* and compared in the intracellular survival assay with p69 and pOLYG as controls (Figure 9). *M. smegmatis* transformants with p69-8, p69-16, and p69-96A all contain a disrupted ORF2 and survived no better than pOLYG transformants. *M. smegmatis* containing p69-96 and p69-97 had levels of intracellular survival comparable to that of p69 and significantly higher than that of pOLYG ($P < 0.001$). The activity of ORF2 in either orientation provides evidence that ORF2 can be expressed from its own promoter. These results confirm that the *M. tuberculosis* gene ORF2 is directly responsible for the enhanced intracellular phenotype associated with p69 in *M. smegmatis*. Therefore, ORF2 was named the enhanced intracellular survival (*eis*) gene.

Efficiency of selection of *eis*-bearing transformants by U-937 cells.

With the identification of *eis*, it became possible to measure its rate of selection during serial passages in U-937 cells. Ten independent clones were selected at random from the 3rd to 6th passages of the *M. smegmatis* transformant pools. Plasmids from these clones were isolated and characterized by digestion with *Bam*HI and *Sma*I to determine the percentage with the same restriction digest patterns as p69. In addition, 50 independent clones were randomly selected from each of these same passages and two *eis*-specific oligonucleotide primers 69.4F and 69.8R (Table 3, see Materials and Methods section) were used in PCR analysis for the presence of the *eis* gene in each clone. Results showed that the *eis*-bearing transformants, undetectable in the 2nd passage, were enriched by the 6th passage to 70 or 88% of the transformant pool, as

determined by restriction digestion or PCR analysis, respectively (Figure 10). Similar results were obtained in a second independent series of passages of the recombinant library in the U-937 survival assay (data not shown).

There is a possible alternative explanation for the enrichment of *eis*-bearing transformants during library passage in U-937 cells. It may be that *eis*-bearing transformants grew faster in the medium used to prepare the inocula for each round of selection in U-937 cells. However, the growth curves of *M. smegmatis*, *M. smegmatis* (pOLYG), and *M. smegmatis*(p69) in 7H9 medium were found to be indistinguishable (data not shown). Therefore, these results show that the intracellular survival assay efficiently enriches for the transformants with enhanced survival phenotypes and that *eis* confers a real survival advantage on *M. smegmatis* containing this gene.

Demonstration of *eis* only in *M. tuberculosis* complex members.

If *eis* contributes to the survival of *M. tuberculosis* in macrophages, it might be present in pathogenic species but absent in nonpathogenic species. The genomic DNA of a number of mycobacterial species was examined by Southern analysis using a PCR-generated DIG-labeled probe to detect the presence of *eis* (Figure 11). The *eis* gene was identified as a 12-kb band present only in *M. tuberculosis* H37Rv, H37Ra, Erdman, and in *M. bovis* BCG. None of the numerous other mycobacterial species tested, including *M. smegmatis*, hybridized with the DIG-labeled *eis*-1 probe. These results demonstrate that *eis* occurs only in pathogenic *M. tuberculosis* complex members and their laboratory-produced derivatives.

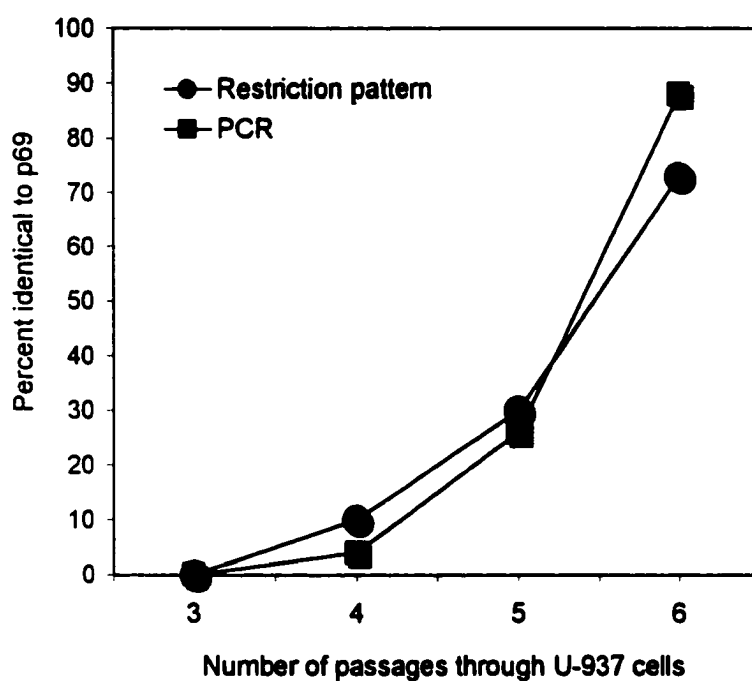


Figure 10. Enrichment of p69 during passage through U-937 cells. Plasmid restriction patterns were analyzed for 10 clones randomly selected from each passage. PCR analysis was performed on 50 clones randomly selected from each passage. Details of the methodology are given in Materials and Methods.

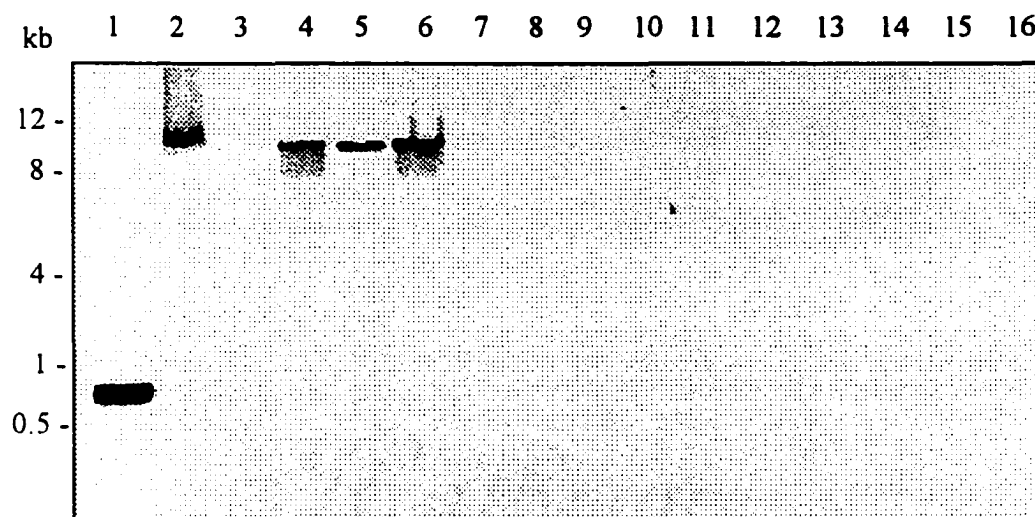


Figure 11. Southern blot analysis for the presence of *eis* in *Mycobacterium* spp. The 824-bp DIG-labeled PCR product from *eis* was hybridized to *Pst*I-digested chromosomal DNA. Lanes: 1, 824-bp PCR product of *eis* using *eis*-probe 1 ; 2, *M. bovis* BCG; 3, *M. smegmatis* 1-2c; 4, *M. tuberculosis* H37Rv; 5, *M. tuberculosis* Erdman; 6, *M. tuberculosis* H37Ra; 7, *M. abscessus*; 8, *M. aurum*; 9, *M. avium*; 10, *M. gordonae*; 11, *M. peregrinum*; 12, *M. phlei*; 13, *M. triviale*; 14, *M. vaccae*; 15, *M. chelonae*; 16, *M. fortuitum*.

Production of the putative *eis* gene product in *M. smegmatis* is correlated to the enhanced intracellular survival phenotype.

To examine whether the *eis* gene could be expressed in *M. smegmatis*, *M. smegmatis* transformants containing vector pOLYG or p69 were lysed by vortexing with glass beads. Proteins from the various cell lysates were separated on a 12% SDS-PAGE gel. Figure 12 shows a unique Coomassie blue-stained band corresponding to a protein matching the predicted size of Eis (42 kDa) in lysates of *M. smegmatis* transformants containing p69, but not in lysates of pOLYG. Therefore this protein band is very likely the *eis* gene product.

M. smegmatis transformants containing deletion derivatives of p69 were also examined for the presence of an *eis* gene product. Figure 13 shows that in the presence of an intact *eis* gene (p69, p69-97, and p69-96), there is observed a unique 42-kDa protein band. In contrast, this protein band is not present in *M. smegmatis* transformants containing *eis* gene deletion constructs (p69-16, p69-8, and p69-96A). Therefore, the appearance of this 42-kDa Eis protein directly correlates with enhanced intracellular survival of the various transformants containing an intact *eis* gene (see Figure 9).

The putative promoter of the *eis* gene.

Systematic studies for identification of mycobacterial promoters have revealed a conserved –10 region with consensus sequence T₈₀A₉₀Y₆₀G₄₀A₆₀T₁₀₀ (where Y is a pyrimidine base) for *M. tuberculosis* (24). In contrast, poor conservation in the –35

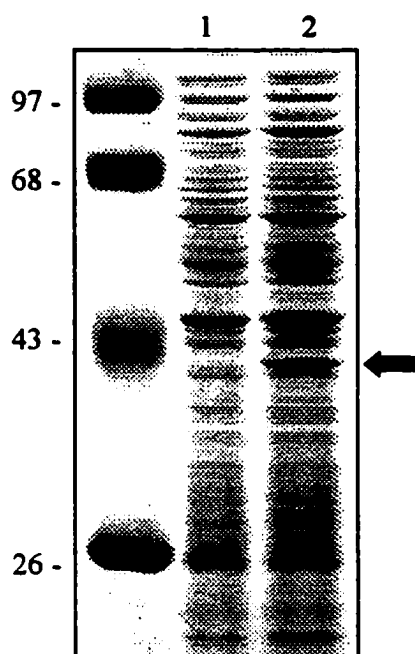


Figure 12. SDS-PAGE analysis of lysates of *M. smegmatis* transformants containing vector alone (pOLYG) (lane 1), or p69 (lane 2). Protein lysates (100 μ g of total protein) were loaded in each lane. A unique Coomassie blue-stained band corresponding to a 42-kDa protein (arrow) is present only in the *M. smegmatis*(p69) lysate, which contains an intact *eis* gene. Protein molecular size standards (in kilodaltons) are shown on the left.

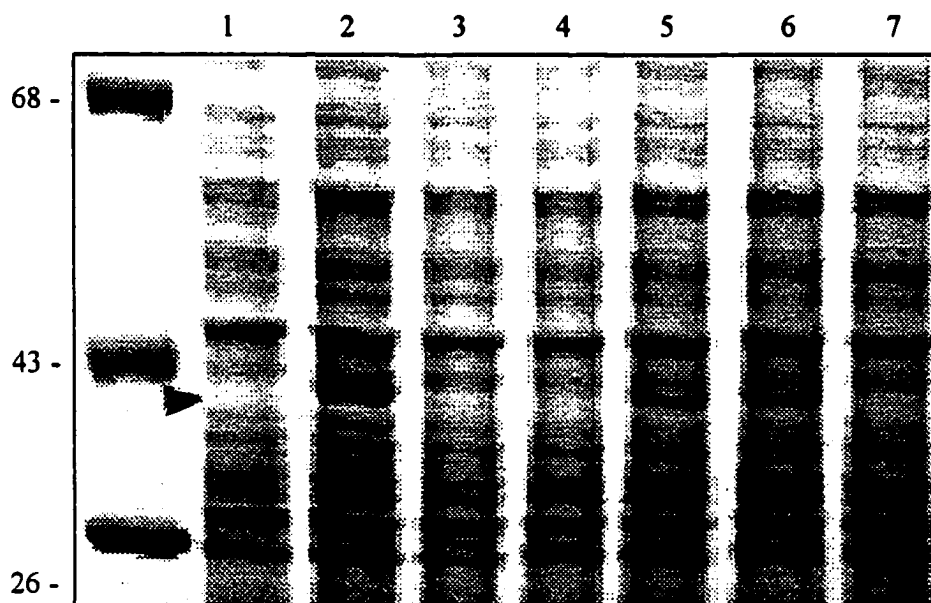


Figure 13. SDS-PAGE analysis of *M. smegmatis* transformants containing vector alone (pOLYG), p69, or deletion derivatives of p69. Mycobacterial lysates (140 μ g of total protein per lane) were subjected to electrophoresis by using a 12 % SDS-PAGE gel. Lane 1-7: *M. smegmatis* containing pOLYG, p69, p69-16, p69-8, p69-97, p69-96, and p69-96A respectively. A unique Coomassie blue-stained band corresponding to a 42-kDa protein (arrow) is present only in *M. smegmatis* transformants containing an intact *eis* gene (p69, p69-97, and p69-96) and not in transformants containing *eis* gene deletions (p69-16, p69-8, and p69-96A). Protein molecular size standards (in kilodaltons) are shown on the left.

region was found (24). This lower strength of mycobacterial promoters was suggested to be related to the high G+C content in the genome (25). The diversity of promoter sequences observed in mycobacteria could also be the result of having multiple σ factors (as many as 13) in *M. tuberculosis* (194). Our sequence analysis has identified a putative promoter region upstream of the *eis* gene with similarity to the -35 consensus TTGACA for *E. coli* σ^{70} -dependent promoters and to the -10 region TAYGAT for *M. tuberculosis* promoters (Figure 6) (311). Table 4 shows the comparison of the putative *eis* promoter sequence and other defined *M. tuberculosis* promoters. Using the constructs containing only the *eis* gene and its putative promoter in opposite orientations (p69-96 and p69-97), we observed very similar results in gene product analysis (Figure 13) and the intracellular survival assay (Figure 9). These data strongly suggest that *eis* is expressed using its own predicted promoter. Yet, further direct experimental evidence such as primer extension or S1 mapping experiments is required to confirm the transcriptional start site (+1) of the *eis* gene.

A putative ribosomal binding site (RBS) was also identified upstream of the *eis* gene (Figure 6). Sequence analysis shows that there are three in-frame start codons at the beginning of Rv2416c by which *eis* could potentially produce either a 408, 402, or 387 amino acid gene products, respectively. We initially proposed that *eis* might use the third ATG for translation because a putative RBS sequence (GAGGA) was identified downstream of the second start codon GTG (311). By sequencing N-terminal amino acids of the Eis protein, Dahl *et al.* recently demonstrated that the *eis* gene is translated using the second GTG as the start codon (75). It was suggested that

Table 4. Promoter sequence comparison of the *eis* gene and various *M. tuberculosis* genes.

Gene ^b	-35	Spacer	-10	Reference
CONSENSUS^a				
<i>E. coli</i>	T₈₂T₈₃G₇₈A₆₄C₅₃a₄₄		T₈₂A₉₀T₅₂A₅₉a₄₉T₈₉	(150)
<i>M. tuberculosis</i>	N/A		T₇₆A₈₁K₆₆R₆₆a₄₂T₈₁	(24)
Mtb_ <i>irgI</i>	<u>TCGACC</u>	16.....	<u>TAGCCT</u>	(257)
Mtb_ <i>fbpA</i>	<u>TACACG</u>	17.....	<u>CGCCTG</u>	(172)
Mtb_ <i>lexA</i>	<u>TTGATT</u>	17	<u>TACATT</u>	(205)
Mtb_ <i>sigH</i>	<u>CTGGAA</u>	20	<u>GTTGAT</u>	(110)
Mtb_ <i>purC</i>	<u>AGCACA</u>	20	<u>TAGGCT</u>	(159)
Mtb_ <i>erp</i>		<u>TAGACT</u>	(33)
Mtb_ <i>eis</i>	<u>TTGCCA</u>	14	<u>TAATAT</u>	(311)

^a Capital letter indicates more than 50% conservation; lowercase letters indicate between 25 % and 50 % conservation K, G or T; R, A or G.

^b *irg*, iron regulated gene

fbpA, fibronectin-binding protein 85A gene

lexA, regulatory gene for SOS functions

sigH, sigma factor H gene

purC, purine synthetase C gene

erp, exported repetitive protein gene

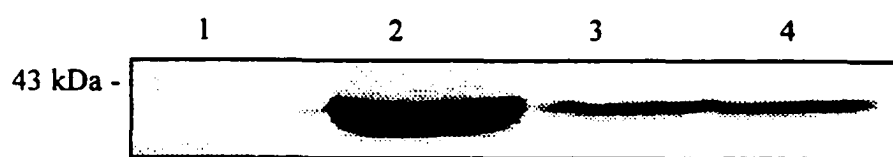


Figure 14. Western blot analysis of lysates of *M. smegmatis* transformants and *M. tuberculosis* for presence of the Eis protein. Mycobacterial lysates (50 μ g of total protein per lane) were subjected to SDS-PAGE, transblotted onto nitrocellulose, and analyzed by Western blot using anti-Eis antibody. Lanes: 1, *M. smegmatis*(pOLYG); 2, *M. smegmatis*(p69); 3, *M. tuberculosis* H37Ra; 4, *M. tuberculosis* H37Rv. The position of a 43-kDa protein molecular size standard is shown on the left.

AGTCGGA act as the RBS sequence for the *eis* gene (Figure 6).

Production of Eis in *M. smegmatis* transformants and *M. tuberculosis*.

The *eis* gene is present only in *M. tuberculosis* H37Rv, H37Ra, Erdman, and in *M. bovis* BCG, but its putative protein product has previously only been shown to occur in *M. smegmatis* containing *eis* on the plasmid pOLYG. For detection of Eis in mycobacterial lysates by Western blot analysis, rabbit anti-Eis antibody was produced by using a synthetic peptide corresponding to the C-terminal domain of Eis as described in Materials and Methods. Results of the Western blot analysis are presented in Figure 14. The Eis protein was readily detected in lysates of both *M. tuberculosis* H37Ra and H37Rv. The anti-Eis antibody also detected the presence of the 42-kDa Eis protein in lysates of *M. smegmatis*(p69), but not in lysates of *M. smegmatis*(pOLYG), since this mycobacterial species does not contain an *eis* homologue. It was observed that the *eis* gene was overexpressed in *M. smegmatis*(p69). In another study, the Eis protein was shown to be overproduced in *M. smegmatis*(p69) at 8-fold higher levels as compared to that in *M. tuberculosis* H37Rv(75). These results demonstrate that the Eis protein is produced in *M. smegmatis*(p69) and that it is also produced and readily detectable in *M. tuberculosis*, its native host cell.

Introduction of a single copy of the *M. tuberculosis eis* gene into *M. smegmatis*.

Studies were done to determine whether the enhanced intracellular phenotype conferred by the *eis* gene on the multicopy plasmid pOLYG in *M. smegmatis* was due

to a gene dosage effect or if enhanced survival would still occur with *eis* present as a single copy in the chromosome. An integrative mycobacterial plasmid pMV306 (287) was used to directly insert a wild-type copy of *M. tuberculosis eis* gene into the *M. smegmatis* chromosome. pMV306 is based on mycobacteriophage L5 (180) and lacks a mycobacterial origin of replication. This plasmid carries the phage attachment site (*attP*), an integrase gene (*int*), and a kanamycin resistance gene for selection of transformants (180). Therefore, the vector can efficiently transform mycobacteria only via integration into a specific chromosomal site (*attB*). The insertion at this site is stable because the excisionase gene is not present for release of the inserted sequence. Using the integrative vector pMV306, a single copy of the *M. tuberculosis eis* gene was inserted into the chromosome of *M. smegmatis* 1-2c and designated *M. smegmatis*(c69). The presence of *eis* in kanamycin-resistant transformants was confirmed by PCR analysis (data not shown). The genotype of c69 was also verified by Southern blot analysis using a PCR-generated *eis*-1 probe (Figure 15). This analysis revealed a 1.5-kb restriction fragment carrying *eis* in kanamycin resistant c69 (lane 2), but not in *M. smegmatis*(cMV306) (lane 1), in which only the vector pMV306 was integrated into the chromosome. This result demonstrated that the *eis* gene was present on the chromosome of *M. smegmatis*(c69).

Western blot analysis was also carried out to confirm the expression of Eis in *M. smegmatis* transformants (Figure 16B). Using the anti-Eis antibody on mycobacterial construct lysates, the Western blot analysis showed that *M. smegmatis*(c69), produced the Eis protein (lane 3) while the control strain (cMV306) did not (lane 4). The Eis

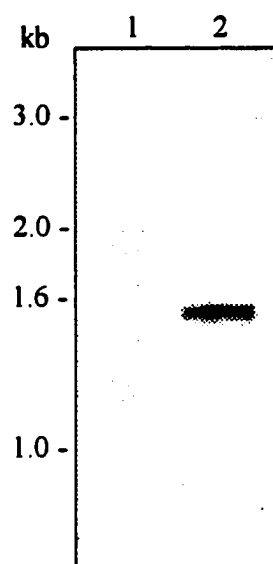


Figure 15. Southern blot analysis for the presence of the *M. tuberculosis eis* gene on the chromosome of *M. smegmatis*. Genomic DNA was isolated from transformants and digested with *Cla*I and *Hind*III. DIG-labeled PCR products of the *eis* gene (*eis*-1 probe) were used as a probe. Lane 1, cMV306 (a construct containing only the vector pMV306 inserted into the chromosomal *attB* site of *M. smegmatis*.); lane 2, c69 (a *M. smegmatis* clone containing a single-copy of *eis* integrated into the chromosomal *attB* site).

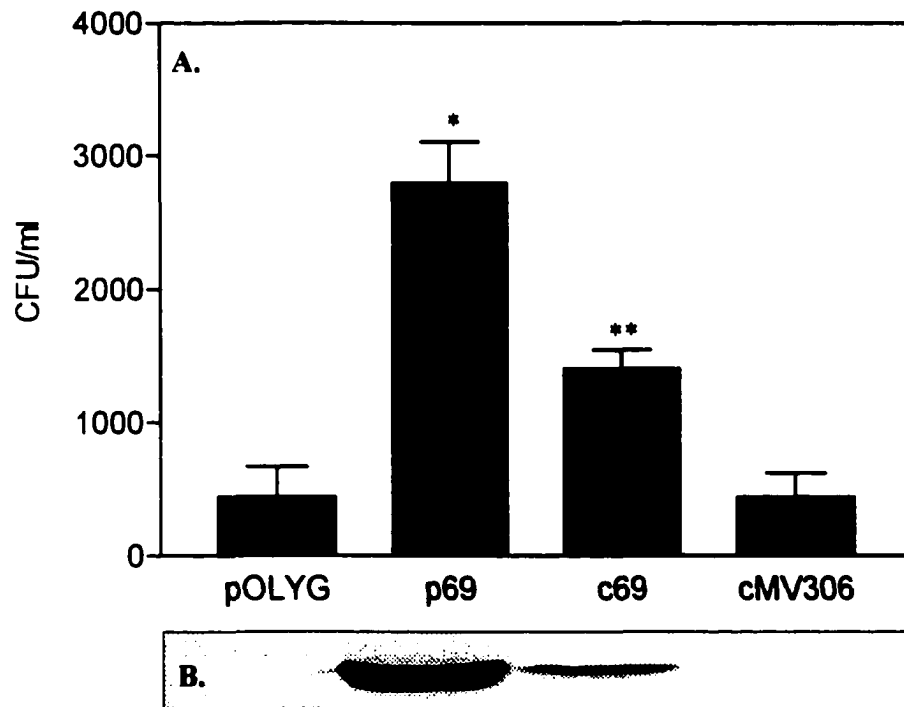


Figure 16. A. Survival in U-937 cells of *M. smegmatis* containing a single chromosomal copy of *eis* (c69) and its vector control (cMV306) as compared to *M. smegmatis*(p69) and its vector control (pOLYG). Surviving intracellular bacteria were counted 48 h after infection. The mean and standard error of three independent experiments are shown.

* $p < 0.0001$ (compared to pOLYG); ** $p < 0.0001$ (compared to cMV306).

B. Western blot analysis of the production of Eis in *M. smegmatis* transformants.

Western blot analysis was done on cell lysates (50 μ g total protein per lane) with the anti-Eis antibody. The order of samples is *M. smegmatis*(pOLGY), *M. smegmatis*(p69), *M. smegmatis*(c69), and *M. smegmatis*(cMV306).

protein is clearly overproduced in *M. smegmatis*(p69) (lane 2), as compared to *M. smegmatis*(c69).

A single copy of the *eis* gene still enhances the intracellular survival of *M. smegmatis*.

To be certain that the enhanced intracellular survival phenotype observed in *M. smegmatis*(p69) was due to the presence of the *eis* gene and not to any possible dosage-effect, it was necessary to show clones with a single copy of *eis* gene still conferred the enhanced intracellular survival phenotype. Figure 16A shows the survival in U-937 cells of *M. smegmatis* containing pOLYG or p69 as compared to single-copy *eis*-bearing *M. smegmatis*(c69) and its vector control (cMV306). It was found that *eis* as a single chromosomal copy in *M. smegmatis*(c69) still demonstrated a 3.2-fold enhanced survival, as compared to the integrated vector control *M. smegmatis*(cMV306). In comparison, *M. smegmatis*(p69) showed a 6.4-fold enhanced survival as compared to its vector control, *M. smegmatis* (pOLYG). Thus the presence of *eis* as a single chromosomal copy in *M. smegmatis* still enhanced its intracellular survival in U-937 cells and suggests that the enhancement by *eis* is not just due to a gene dosage effect.

Isolation of *eis* knock-out and complemented mutants in *M. tuberculosis* H37Ra and H37Rv.

To examine the role of Eis in *M. tuberculosis*, a mutant defective in the *eis* gene and a complemented mutant were constructed. The *eis* gene was disrupted in both *M.*

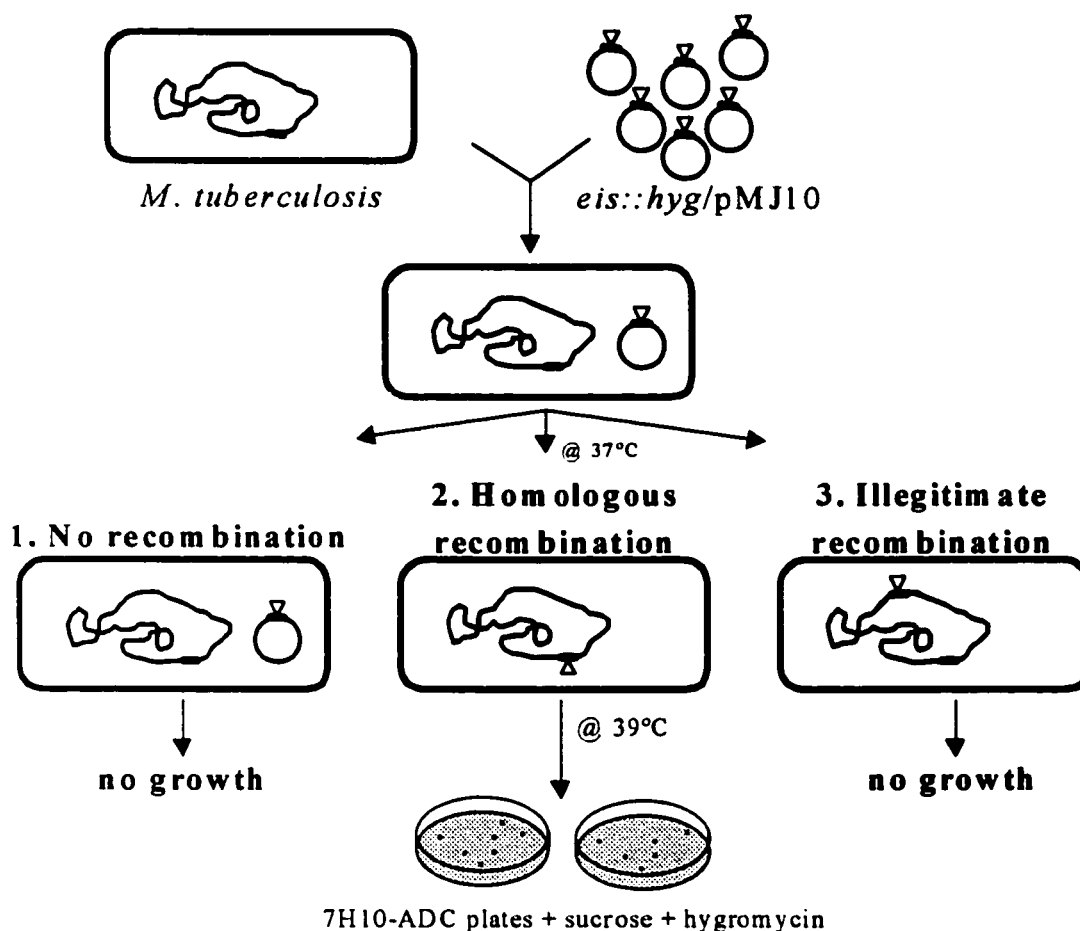


Figure 17. Two-step counter-selective strategy for isolation of double-cross-over events leading to allelic replacement of the *eis* gene. Constructs of pMJ10 containing the *eis::hyg* allele were introduced into *M. tuberculosis* cells by electroporation. Hygromycin-resistance clones were obtained, and cultured in 7H9-ADC broth for 2 d at the permissive temperature of 37°C. During the incubation: (1) the vector construct could remain extra-chromosomal, (2) homologous recombination-mediated double cross-over between the *eis* gene on chromosome and *eis::hyg* on plasmid could result in allele replacement, and (3) the plasmid could integrate into the chromosome by a single cross-over event (illegitimate recombination) to generate a merodiploid. Since plasmid pMJ10 carries a temperature-sensitive origin of replication for mycobacteria and *sacB* counter selectable markers, selection for sucrose-resistance and growth at 39 °C should only isolate clones with allele replacement and loss of vector sequence.

tuberculosis H37Ra and H37Rv by a two-step gene replacement strategy (Figure 17) (237). An allelic exchange vector, *eis::hyg/pMJ10*, which contains the *eis* gene disrupted by a hygromycin cassette, was constructed. After electroporation, twenty hygromycin-resistant merodiploid clones were recovered from both H37Ra and H37Rv. Two independent isolates were randomly selected from each and cultured in 7H9-ADC broth at the permissive temperature of 37 °C for 2 d. During the incubation, three possible genetic events could occur. First, the vector construct may still remains extra-chromosomal; second, a homologous recombination (double cross-over) event leads to the replacement of the wild-type *eis* gene with the disrupted allele (*eis::hyg*); and third, an illegitimate recombination (single cross-over) event leads to random integration of the entire plasmid into the chromosome. Since this vector contained the counter-selectable *sacB* gene (238), which confers sucrose sensitivity, and the temperature-sensitive mycobacterial origin of replication, these merodiploids were subjected to second-step selection by plating and incubating the cultures on 7H10-OADC agar containing hygromycin and 2 % sucrose at 39 °C for counterselecting for the loss of the vector. Clones capable of growth on sucrose plates at 39 °C were anticipated to have only the *eis::hyg* allele on the chromosome by double cross-over. After three weeks, a total of eighty colonies that grew on sucrose-containing plates were picked and restreaked on this media to confirm the stability of these potential allelic exchange mutants. Ten of them were picked for further characterization.

Once the disruption of *eis* gene was confirmed (Figure 18, 19, and 20), the H37Ra and H37Rv *eis* knock-out mutants were complemented using the integrative

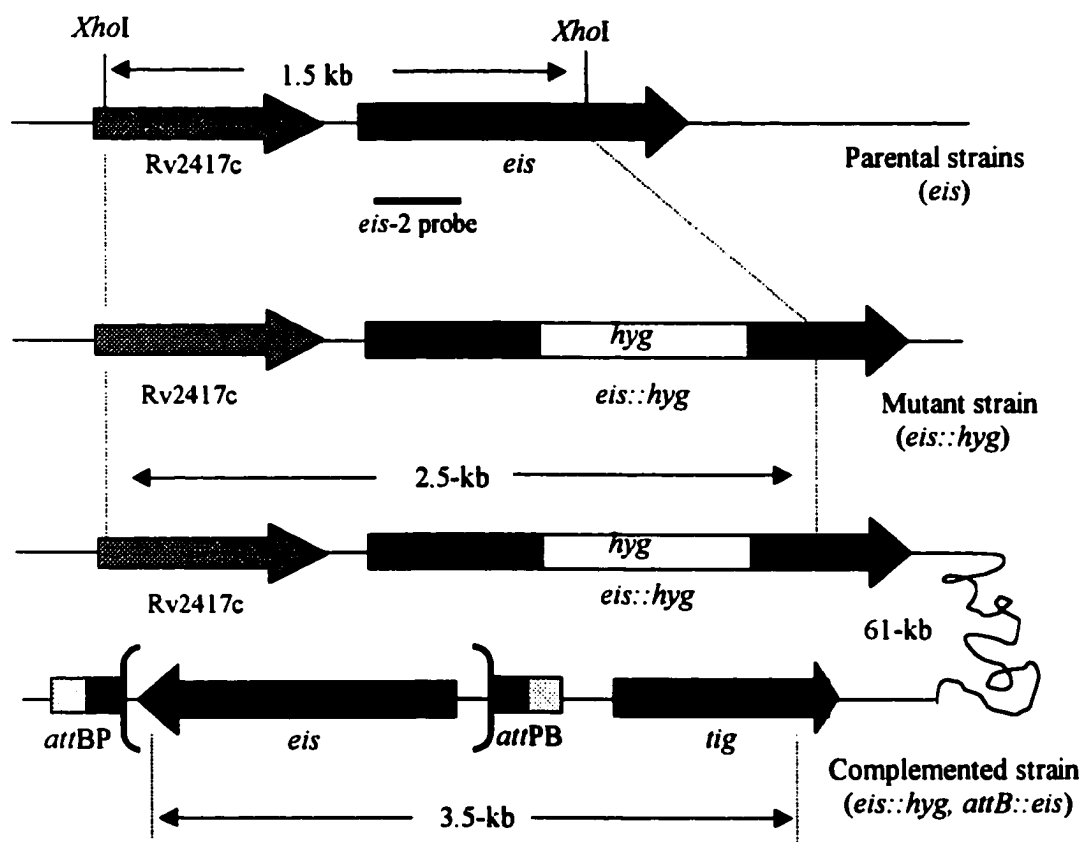


Figure 18. Chromosomal organization of the *eis* gene in parental, mutant, and complemented strains of *M. tuberculosis* H37Rv and H37Ra. *attB*, mycobacteriophage chromosomal integration site; *tig*, gene for trigger protein-like chaperone protein.

mycobacterial vector p6301 containing a wild-type copy of the *eis* gene. Construct p6301 was introduced into *eis* mutants by electroporation. Since this vector carries a kanamycin resistance cassette but does not contain a mycobacterial origin of replication, kanamycin-resistant transformants could only arise if the plasmid becomes incorporated into the mycobacterial chromosome *attB* site (287). After three-week incubation, twenty double-resistant colonies that grew on 7H10-OADC plates containing kanamycin and hygromycin were recovered for confirmation of complementation.

Confirmation of the *M. tuberculosis eis* mutant and complemented strains by Southern and Western blot analysis.

Ten potential *eis* knock-out H37Ra and H37Rv mutants were analyzed by Southern blot analysis. Chromosomal DNA was isolated from both parental and potential *eis* knock-out mutants in H37Ra and H37Rv, digested with *Xho*I, and probed with DIG-labeled *eis*-2 probe for the detection of the *eis* gene. Figure 18 shows the expected *eis*-bearing DNA fragments in parental, mutant, and complemented strains of *M. tuberculosis*. The *eis* gene is detected as a 1.5-kb band present in both parental H37Ra and H37Rv (Figure 19 A and B, lane 1). With the integration of the *hyg* cassette (1.3-kb) into *eis*, the mutated *eis* band now migrates as a larger 2.5-kb band in both the H37Ra and H37Rv *eis* knock-out mutants (Figure 19 A and B, lane 2). With the introduction of the wild-type allele of *eis* the complemented strains, by Southern blot analysis, now have two *eis* reactive bands, one at 2.5-kb (*eis::hyg*) and an additional band at 3.5-kb (*eis::attB*)

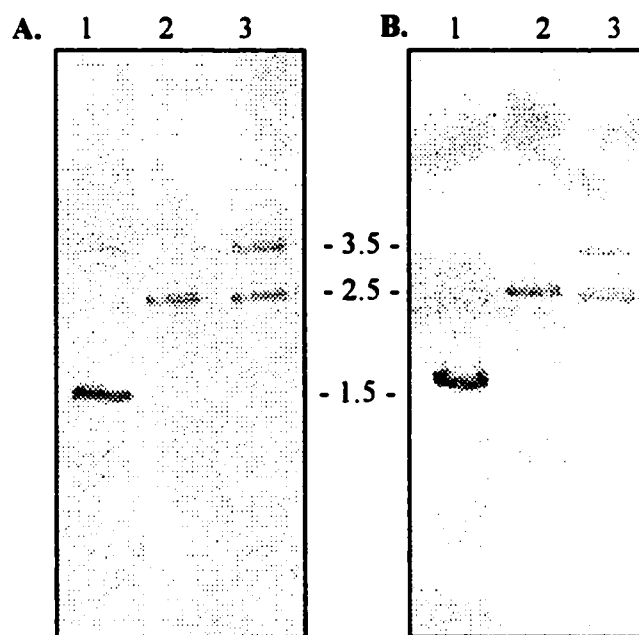


Figure 19. Southern blot analysis of chromosomal DNA from the parental, mutant, and complemented strains of *M. tuberculosis* H37Ra (A) and H37Rv (B). Genomic DNA was digested with *Xho*I and probed for hybridization with DIG-labeled *eis*-2 probe. Molecular masses are indicated in kilobases. The *eis* hybridizing fragment in mutant (lane 2) is ~1.0 kb larger in size than in the parental strain (lane 1). As expected, an additional 3.5-kb band was detected in the complemented strain (lane 3) which corresponds to the DNA fragment containing the complementing copy of *eis* (*eis::attB*).

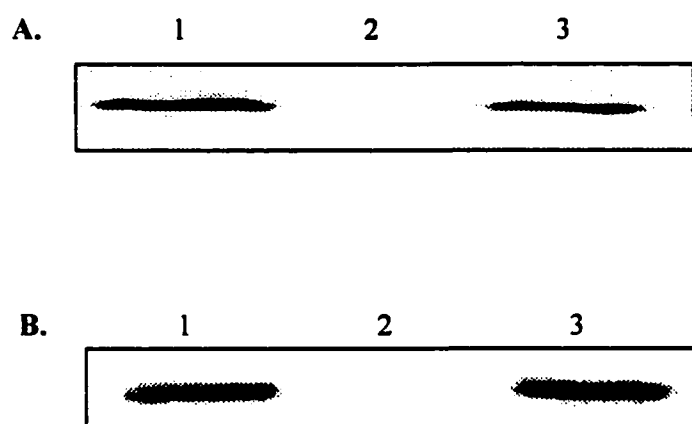


Figure 20. Production of the Eis protein in parental, mutant, and complemented strains of *M. tuberculosis* H37Ra (A) and H37Rv (B). Western blot analysis was done on cell lysates of 3 week old cultures of parental (lane 1), mutant (lane 2), and complemented (lane 3) strains using anti-Eis antibody. Fifteen micrograms of total protein were loaded on each lane.

(Figure 19 A and B, lane 3). Identical results were observed for the complemented mutants in both H37Ra and H37Rv.

The production of Eis protein in parental *M. tuberculosis*, *eis* knock-out mutant, and complemented strains was investigated by Western blot analysis using anti-Eis antibody. As shown in Figure 20, the Eis protein, corresponding to a 42-kDa protein, was found in the lysates of parental strains (Figure 20 A and B, lane 1), but not in the *eis* knock-out mutants in which the native *eis* gene is disrupted (Figure 20 A and B, lane 2). In contrast, strains complemented with the reintroduction of *eis* at the *attB* site of the *eis* mutants regained the production of Eis to levels similar to that of the parental strains (Figure 20 A and B, lane 3).

The results of both the Southern and Western blot analysis verify that *eis* knock-out and complemented mutants in both *M. tuberculosis* H37Ra and H37Rv have been successfully constructed.

***In vitro* growth studies.**

To examine whether the inactivation of *eis* had an effect on *in vitro* growth and survival, a comparison of the growth rate of mutant and parental strains of *M. tuberculosis* H37Ra and H37Rv in both enriched Middlebrook 7H9-ADC and synthetic GAS medium was done. The results for *M. tuberculosis* H37Ra and its *eis* knock-out mutant are shown in Figure 21. Both strains grew equally well with similar doubling times (19 h in 7H9-ADC medium (A) and 20 h in GAS medium (B)). The parental, *eis* knock-out mutant, and complemented strains of *M. tuberculosis* H37Rv also exhibited

the same growth rate in both the synthetic medium and enriched medium (Figure 22). Examination of the colony morphology of the *eis* mutants and parental strains showed that *eis* mutation did not affect the typical rough texture of the strains (data not shown). Taken together, these data indicate that *eis* is not essential for the *in vitro* growth of *M. tuberculosis* H37Ra and H37Rv.

Effect of the *eis* knock-out mutation on the intracellular growth of *M. tuberculosis*.

Both *M. tuberculosis* virulent strain H37Rv and avirulent strain H37Ra have been demonstrated to grow in human monocyte-derived macrophages (234), but H37Rv grows more rapidly intracellularly than H37Ra (328). The similar result was observed in U-937 cells (data not shown). To examine the consequences of loss of *eis* on the intracellular growth of *M. tuberculosis* H37Rv and H37Ra, the multiplication capacity of parental, *eis* knock-out mutant, and the complemented strains in human macrophage-like cell line U-937 cells were analyzed.

Figure 23 shows the growth of *M. tuberculosis* H37Ra, the *eis* knock-out mutant, and the complemented strain in U-937 cells. U-937 monolayers were infected for 4 h with these strains at an MOI of 1. Intracellular bacteria were recovered 2, 4, and 6 days after infection by lysing the monolayers with 1 % Triton X-100 to estimate the numbers of CFUs. The number of intracellular H37Ra CFUs increased 6- to 8-fold over the 6 d of the assay. Analysis of CFUs indicated that the *eis* mutant Ra610 multiplied as well as the parental H37Ra, and the complemented strain Ra610-15 over the 6 days of the experiment (Figure 23). These results suggest that the intracellular growth potential of

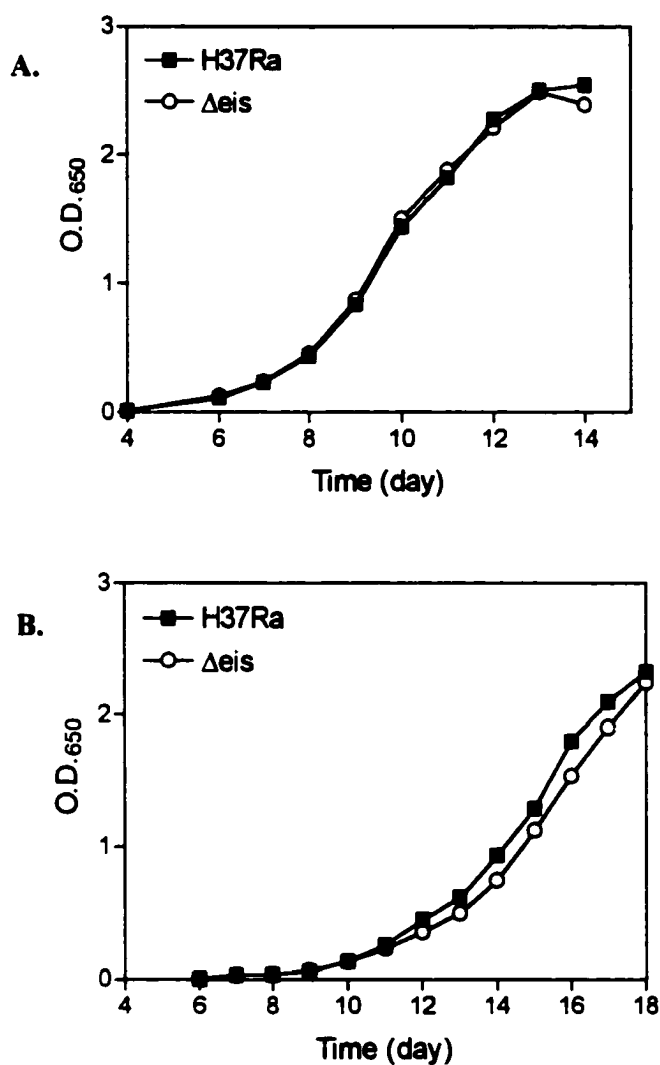


Figure 21. Growth of *M. tuberculosis* H37Ra and *eis* mutant (Δeis) in enriched 7H9-ADC medium (A) and in synthetic GAS medium (B). Approximately 2×10^5 bacteria were inoculated into 50 ml of each medium with appropriate antibiotics. O.D.₆₅₀ was measured every 1 to 2 d. The results presented are from a representative experiment.

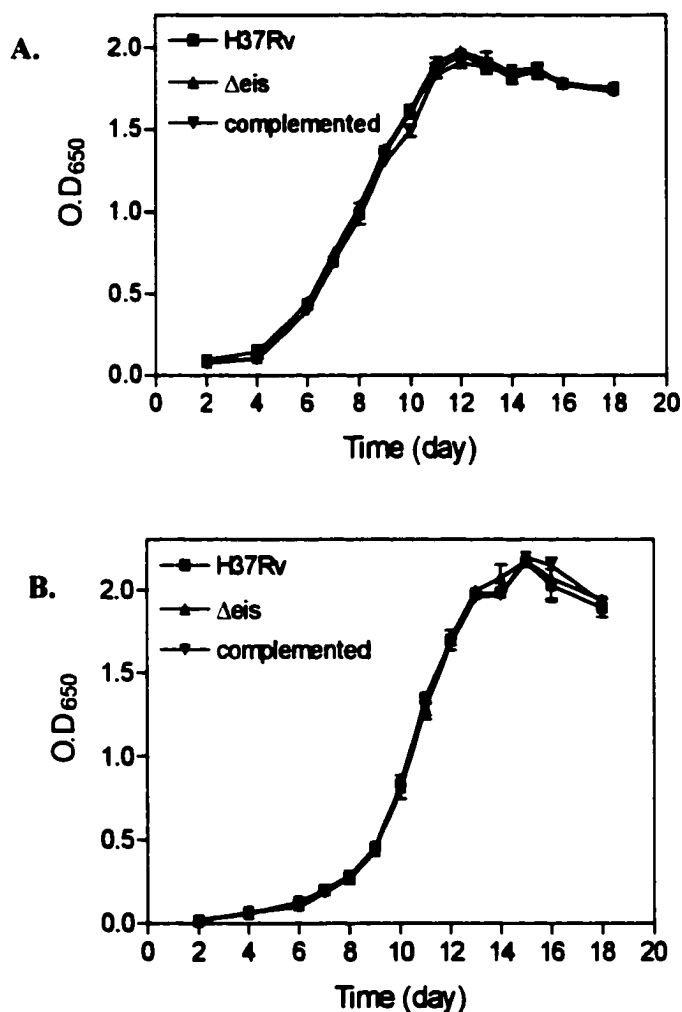


Figure 22. Growth of parental *M. tuberculosis* H37Rv, *eis* mutant (Δeis), and complemented strains in enriched 7H9-ADC medium (A) and in synthetic GAS medium (B). Approximately 2×10^5 bacteria were inoculated into 50 ml of each medium with appropriate antibiotics. O.D.₆₅₀ was measured every 1 to 2 d. Data presented are the means \pm standard errors of duplicate samples from a representative experiment.

the H37Ra *eis* mutant is not impaired as compared to its parental strain.

The growth of *M. tuberculosis* H37Rv, its *eis* knock-out mutant, and the complemented strain in U-937 cells was analyzed over a 7-d period. Using the same procedure as for H37Ra (MOI=1), CFUs recovered from the 1 % Triton X100 lysates of U-937 monolayers at each time point were found to be lower than expected (data not shown). Acid-fast stains of U-937 monolayers showed that both the *eis* mutant- and parental H37Rv-infected macrophages started to undergo extensive lysis after 2 d of infection. Adherent cell counts indicated that more than 70 % the U-937 cells were lost after 4 d of infection (data not shown). This was not observed with H37Ra, which suggested that H37Rv grew faster than H37Ra within U-937, caused rapid lysis of the macrophages, and resulted in decreased numbers of adherent U-937 cells. Therefore, macrophage lysis resulted in the release of intracellular mycobacteria into the tissue culture medium. To solve this problem, total lysates of infected U-937 cells, including both media and lysed macrophages, were used for CFU analysis by adding $1/10$ volume of 10 % Triton X-100 directly into the medium. Both intracellular mycobacteria and those already being released into the medium were present in total lysates. It was demonstrated that H37Rv did not grow in RPMI-10 % human serum or in RPMI-10 % human serum supplemented with macrophage lysates (data not shown). Because *M. tuberculosis* H37Rv did not grow extracellularly in the medium, any increase of numbers of mycobacteria in the total lysates should be due to intracellular bacterial growth. Figure 24A shows the intracellular growth of H37Rv strains within U-937 cells. No significant difference was observed between intracellular growth of H37Rv

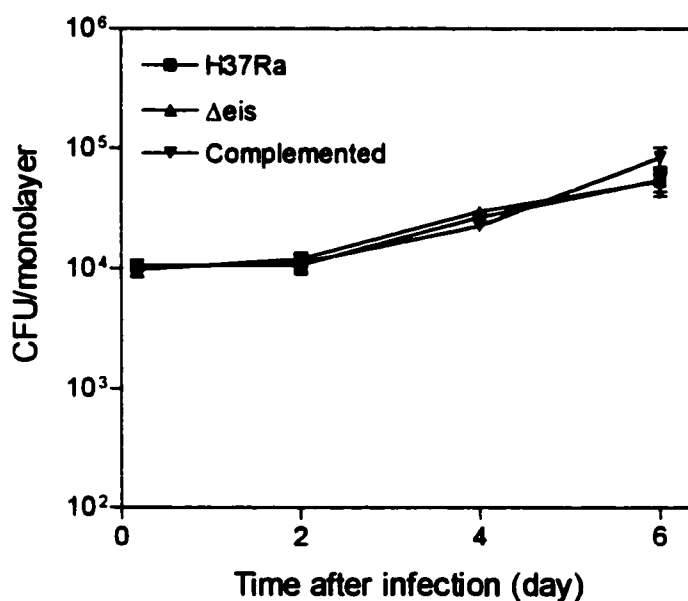


Figure 23. Growth of *M. tuberculosis* H37Ra, *eis* knock-out mutant, and the complemented strain in the human macrophage-like cell line U-937. The U-937 monolayers were infected with parental H37Ra, the *eis* mutant, or the complemented strain for 4 h at the MOI of 1, as described in Materials and Methods. On days 0, 2, 4, 6, the monolayers were lysed, and plated for mycobacterial CFU counts on 7H10-OADC agar with appropriate antibiotics. Mean CFUs \pm standard deviations from 3 independent experiments done in duplicate are shown.

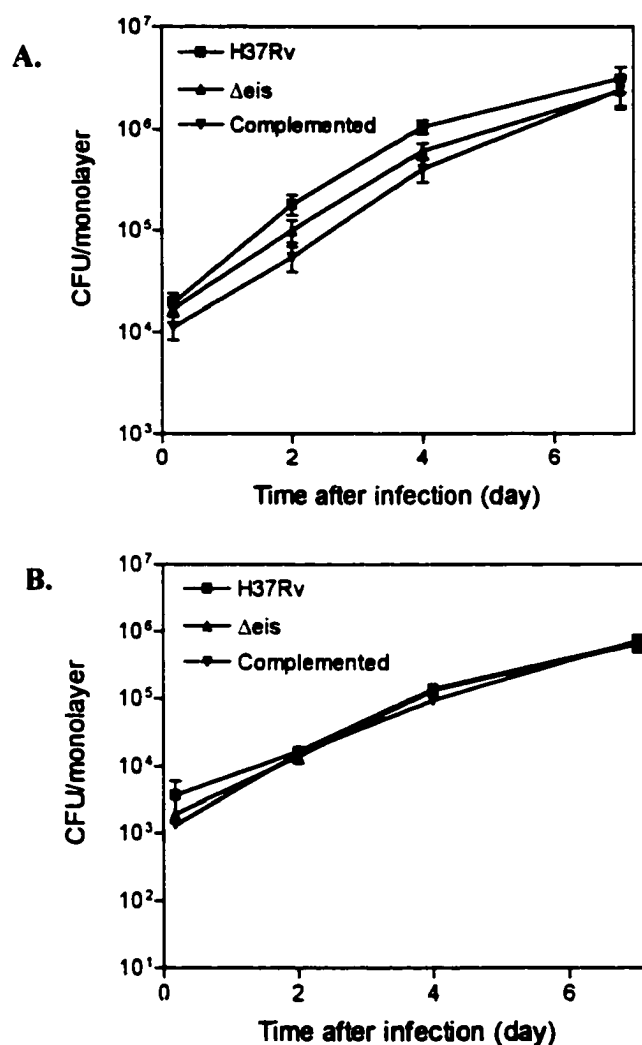


Figure 24. Growth of *M. tuberculosis* H37Rv, its *eis* knock-out mutant, and the complemented strain in human macrophage-like cell line U-937. The U-937 monolayers were infected with H37Rv, the *eis* mutant, or complemented strain for 4 h at the MOI of 1 (A) or 0.1 (B), as described in Materials and Methods. On days 0, 2, 4, 7, the total lysates were plated for mycobacterial CFU counts on 7H10-OADC agar with appropriate antibiotics. Mean CFUs \pm standard deviations for 3 independent experiments done in triplicate are shown.

and that of its *eis* mutant, and the complemented strain.

To reduce the rapid loss of U-937 cells in monolayers infected with H37Rv, a lower MOI of 0.1 was used to evaluate the survival of H37Rv strains in U-937. H37Rv, the *eis* mutant, and the complemented strain all exhibited similar growth rates (Figure 24B). Although a slight decrease in U-937 adherence was observed, CFUs from both lysates of monolayers along and total lysates were similar at each time point (data not shown). These results confirmed that it is valid to use total lysates for CFU analysis in this assay system. Overall, the data presented here suggest that the disruption of the *eis* gene did not limit the intracellular grow or survival of H37Rv and H37Ra in U-937 cells.

DISCUSSION

Understanding the basis of mycobacterial virulence depends on the identification of genes and gene products that contribute to pathogenesis. Using nonpathogenic surrogate hosts for gene expression is one of the techniques successfully used for the study of *M. tuberculosis* virulence genes. Previous workers have used this approach to look for mycobacterial genes needed for survival in macrophages. Mundayoor and Shinnick passed recombinant DNA libraries of *M. leprae* in *E. coli* through a mouse macrophage-like cell line to enrich for clones with increased survival in those host cells (207). Using pOLYG as a cloning vector, Wieles *et al.* cloned the thioredoxin-thioredoxin reductase gene of *M. leprae* into *M. smegmatis* and showed that the transformant was less rapidly killed by human mononuclear phagocytes than *M. smegmatis* with pOLYG alone (315). Arruda *et al.* cloned DNA from avirulent *M. tuberculosis* H37Ra and isolated the *mce* gene, which augmented the ability of *E. coli* to enter and survive in nonphagocytic HeLa cells (8). Ehrt *et al.* screened a plasmid genomic DNA library of a *M. tuberculosis* clinical isolate CB 3.3 in acidified nitrate and identified a clone that enhanced survival of *E. coli* and *M. smegmatis* in macrophages. Their study led to identification of the *noxR1* gene that may be involved in protecting *M. tuberculosis* against ROIs and RNIs (95).

In the present study, the human macrophage-like cell line U-937 was used to screen an *M. tuberculosis* H37Rv gene library. A clone (p69) of avirulent *M. smegmatis* transformed with DNA from the virulent *M. tuberculosis* strain H37Rv significantly

enhanced intracellular survival for at least 48 h in both U-937 cells and human blood monocytes (Figure 1 and 4). Evidence presented here indicates that the prolonged survival of this clone in U-937 cells resulted from the presence of a *M. tuberculosis* gene, designated *eis*, and that disruption of this gene was followed by loss of the enhanced intracellular survival phenotype in *M. smegmatis*.

To confirm that the enhanced intracellular survival phenotype conferred by the *eis* gene on the multicopy plasmid pOLYG was not due to a gene dosage effect, the survival of *M. smegmatis* containing a single copy of *eis* integrated into chromosome was also compared to that of *M. smegmatis*(p69) and *M. smegmatis*(cMV306) in the macrophage survival assay. The macrophage infection still elicited a survival difference between single-*eis*-bearing *M. smegmatis* and its vector control (Figure 16). The increased survival in U-937 cells conferred upon *M. smegmatis* by the presence of single-copy *eis* is modest but still significant, considering that it is the result of introducing a single gene from a highly pathogenic bacterium into a nonpathogenic relative. These findings further confirm that the *eis* gene does enhance *M. smegmatis* intracellular survival in macrophages and indicates that using *M. smegmatis* as a surrogate host to screen for *M. tuberculosis* genes which are important in macrophage intracellular survival, is a valid system which is specific and yields genuine positive clones.

The *eis* gene exhibited a limited distribution within the mycobacteria. Southern blot analysis under high stringency showed that the *eis*-hybridizing sequences were detected only in members of the *M. tuberculosis* complex, and not in any of the seven fast-

growing including *M. smegmatis* and four slow-growing non-tuberculosis mycobacterium species tested (Figure 11). Sequence comparison studies also indicated that *eis* is not present in the genome of pathogenic *M. leprae* (http://www.sanger.ac.uk/Projects/M_leprae/) and that of *M. avium* (http://www.sanger.ac.uk/Projects/M_bovis/). Although not all mycobacterial species were examined, our data suggest that the *eis* gene is restricted to the *M. tuberculosis* complex. Some genes which are limited to slow-growing mycobacteria have been found important to the virulence of *M. tuberculosis*. The sigma factor F (SigF) of *M. tuberculosis*, which is present only in slow-growing mycobacteria (231), was recently demonstrated to be involved in long-term survival of *M. tuberculosis* in a mouse model (55). The *M. tuberculosis* *erp* gene, which is restricted to mycobacteria causing tuberculosis (186) and leprosy (56), was found required for the multiplication of the pathogen within murine macrophages (32). The *mgt* gene of *M. tuberculosis*, a homologue of the *Salmonella* *mgt* gene, is also only present in slow-growing mycobacteria (45). The *mgt* gene has also been shown to be essential to the intracellular survival of *M. tuberculosis* within macrophages (45). The fact that *eis* is found exclusively in mycobacteria causing tuberculosis suggests that the *eis* gene may play a role in the pathogenesis of tuberculosis. A recent study of insertion sequences by Gordon *et al.* has revealed the occurrence of horizontal DNA transfer in the *M. tuberculosis* complex, showing that *M. tuberculosis* can exchange genetic information with other bacteria (136). Limited distribution of *eis* implies that the *eis* gene is not ancestral to all mycobacteria, and may have been acquired by the *M. tuberculosis* complex after their divergence from other mycobacterial species (136).

Identification of *eis* using *M. smegmatis* and U-937 cells suggests the potential of this system for identifying additional genes contributing to the survival of virulent mycobacteria in macrophages. Of the clones isolated from the 3rd passage of *M. smegmatis* transformants in U-937 cells, only p69 has been characterized in detail. There were 20 additional clones initially identified with a 2-fold or greater enhanced intracellular survival in macrophages. Further analysis by restriction digestion, Southern blot, and PCR of these clones showed that virtually all contained the *eis* gene and were similar to p69 (data not shown). Thus all clones selected from the third passage demonstrating enhanced intracellular survival in our U-937 survival assay are not false positives, but true positive clones containing *eis* with an authentic enhanced intracellular phenotype. These findings are also strong evidence that *eis* has a major influence on the survival of *M. smegmatis* in U-937 cells (see Figure 10). Since *eis*-containing clones are preferentially selected and appear to out-compete/dominate other *M. tuberculosis* genes which may also play a role in intracellular survival, work is in progress to prepare a new *M. tuberculosis* plasmid library with genomic DNA from a H37Rv *eis* knock-out mutant to possibly identify other potential genes.

One limitation of this system is the size of the H37Rv DNA insert used in this library, 5.4 kb on average. This effectively limits the probability of finding more than one gene of interest in a single DNA insert. In fact, the insert of p69 is 2.99-kb in size, and contains only *eis* and other two ORFs (Figure 8). Sequence analysis of the *M. tuberculosis* genome suggests that *eis* could use its putative promoter to be transcribed with up to six downstream ORFs as an operon (Figure 7), although none of the other

potential ORFs have known functions in this 9-kb region. It is possible that cosmid libraries of *M. tuberculosis* DNA, with insert sizes of up to 40-kb, may be used to identify virulence factors that require the concerted action of several separate genes or the presence of an entire operon.

A 42-kDa protein has been identified as the *eis* gene product, Eis. Three lines of evidence support this conclusion. First, the unique size of the protein matches the size predicted for an *eis* gene protein product. Second, the appearance of the 42-kDa protein in *M. smegmatis* transformants containing an intact *eis* gene directly correlates with the enhanced intracellular phenotype conferred by the *eis* gene in *M. smegmatis* (Figure 13). Third, anti-Eis antibody recognized the 42-kDa protein in both *M. smegmatis*(p69) and *M. tuberculosis* (Figure 14). Examination of the deduced amino acid sequence of Eis with appropriate computer programs such as BLAST, psi-BLAST and FASTA predicted that the Eis protein would be mainly hydrophilic, with one or two transmembrane regions in its N-terminus and that it would not contain a secretion signal sequence (75). Unfortunately, amino acid sequence analysis did not identify any known homologs to Eis, provided few clues as to its possible function.

Recently Dahl *et al.* demonstrated in *M. tuberculosis* fractionation studies that Eis appears primarily in the cytoplasm, in modest amounts in the cell envelope, and also in the culture supernatant (75). Although it is still not clear how the Eis protein, which lacks a signal sequence, is released into the culture supernatant, this finding suggests that Eis might be exported or secreted. One of the potential biological significances of secreted proteins from *M. tuberculosis* is that these proteins may play a role in

pathogenesis by directly interacting with the host cells. Mycobacterial proteins that are exported intracellularly may participate in phagosome remodeling. The exported repetitive protein (Erp) is secreted by *M. tuberculosis* (33). Like Eis, the Erp protein also has no homology to proteins with known functions and its mode of action and function remains unclear. Interestingly, an *erp* knockout mutant is significantly impaired for survival in macrophages and in immunocompetent mice (32).

Another biological significance of exported *M. tuberculosis* proteins is that they may generate a humoral immune response to the bacilli. Antibodies produced against secreted antigens may have a serodiagnostic function (77). Dahl *et al.* has shown in preliminary studies that 40% of sera from patients with tuberculosis generate antibody against Eis, whereas none of five sera from PPD-positive individuals did (75). However, little attention has been paid to a possible role for antibody in protection from *M. tuberculosis* infection. There are several arguments against a role for humoral immune defense against tuberculosis infections (48). First, *M. tuberculosis* resides intracellularly, and is therefore inaccessible to antibodies. Second, immunization with heat-killed bacilli generates antibodies to *M. tuberculosis* antigens but fails to protect as compared to live bacilli, which induce cell-mediated immunity. Finally, titers of antibodies to *M. tuberculosis* were found to increase with disease progression (48). Recently, accumulative evidence has suggested that humoral immunity can mediate protection against many intracellular pathogens (48). Although *M. tuberculosis* clearly resides within alveolar macrophages at early stages of infection, bacilli can be found extracellularly within tissues in necrotizing granulomas and therefore, in potential

contact with antibodies. Antibodies may also have access to tubercle bacilli before bacterial seeding of the lung and may serve to limit initial infection (48). To determine whether the Eis-elicited antibody is involved in the immunity against tuberculosis, more immunopathology studies need to be done.

Still unanswered is the question of how Eis acts to prolong the survival of *M. smegmatis* and ultimately that of *M. tuberculosis*, in macrophages. Numerous mycobacterial activities must be required for long-term survival of these organisms in human phagocytes (177). First, there are housekeeping activities necessary for optimal growth and survival of mycobacteria in any environment. While mutations in genes encoding enzymes of biosynthetic pathways, such as the *M. tuberculosis* leucine auxotroph (*leuD*) (196) and *purC* mutant (12, 160), have been found to render *M. tuberculosis* avirulent in animal models, these genes are also required for *in vitro* growth of *M. tuberculosis*. The observation that *M. tuberculosis eis* knock-out mutants grew as well as their parental strains in both enriched and synthetic medium makes it unlikely that *eis* is a housekeeping gene (Figure 21 and 22). Second, activities are required to defend the mycobacteria against stressful conditions such as low pH, oxidative stress, and temperature shift. Both pathogenic and non-pathogenic mycobacteria may share some of these activities, such as heat shock proteins (HSPs) (146) in resisting hostile environments. Therefore, these activities may not be specifically involved in pathogenicity. The finding that *eis* is present only in virulent mycobacteria and their laboratory-generated derivatives and not in *M. smegmatis* argues against this possibility. However, *M. tuberculosis* may also possess activities

specific for this organism to resist the stresses inherent within human phagocytes. Introduction of these *M. tuberculosis*-specific activities could enhance nonpathogenic mycobacterial intracellular survival under the stressful conditions encountered in macrophages. Production of reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) is the major nonspecific antimycobacterial mechanisms of macrophages (117). The *noxR1* (95) and *noxR3* genes (260), which may be involved in defence against these chemically reactive micromolecules, have been cloned from *M. tuberculosis*. They were found to confer enhanced resistance to RNIs and ROIs upon *E. coli*, *M. smegmatis*, and *S. typhimurium* (95, 260). Further studies are planned to evaluate *M. smegmatis*(p69), and *M. tuberculosis eis* mutants under different stress conditions for resistance to ROIs and RNIs.

The third class of required activities are those specifically evolved to promote persistence of slow-growing mycobacteria in host cells for long-term survival. *M. tuberculosis* genes involved in these activities have been recently identified. One of the glyoxylate shunt enzymes of *M. tuberculosis*, isocitrate lyase (Icl), was found to be activated during the metabolic downshift that accompanies oxygen withdrawal in an *in vitro* model of latency (138, 157, 288). Studies have shown that inactivation of the *icl* gene impairs mycobacterial survival during late-stage infection but not during the acute phase in mice (198). This suggests that *icl* is required for mycobacterial persistence in the host. Another study showed that inactivation of *sigF* caused no difference in short-term intracellular growth within human monocytes as compared to the wild type parent. In contrast, loss of *sigF* reduced the virulence of *M. tuberculosis* for mice, indicating

that *sigF* might be required for the long-term *in vivo* survival (55). While these activities appear restricted to slow-growing mycobacteria, the effects of transforming these persistence genes into *M. smegmatis* have not been evaluated. To test if the *eis* gene is involved in the persistence of *M. tuberculosis*, long-term *in vivo* studies with the *eis* mutant and wild-type *M. tuberculosis* need to be carried out. The availability of *M. tuberculosis* H37Rv, its *eis* mutant, and complemented strains will allow for testing of this hypotheses in mouse models of both persistence and acute infection. These experiments are presently in progress and are beyond the scope of this dissertation.

Based on Koch's molecular postulates for virulence (102), once candidate virulence genes are identified, it is common to construct isogenic strains of organism that differ only in the expression of the target gene, usually achieved by allelic exchange. Then the loss of virulence needs to be clearly established *in vitro* and *in vivo*. Until recently, the inactivation or mutation of genes in a targeted fashion has been very difficult in slow-growing mycobacteria. By utilizing a suicide vector strategy, homologous recombination leading to allele replacement has been successfully achieved in *M. bovis* and *M. tuberculosis* (10, 12, 237). Several groups have demonstrated the use of *sacB* suicide systems for target disruption of the *erp* (32), *recA* (229), and *sigF* (55) in *M. tuberculosis*. The frequency of double-cross-over events appears to occur at a variable range of 10^{-6} - 10^{-3} (235). To increase the frequency of detection of double-cross-over events, Pelicic *et al.* constructed a double counters selectable suicide vector containing *sacB* and a thermosensitive mycobacterial origin of replication (*ts*). Using this allelic

exchange vector, they mutagenized the *purC* gene of *M. tuberculosis* with efficiencies of 10^{-7} (237).

In this study, the double counterselection strategy was used to successfully disrupt the *eis* gene in *M. tuberculosis* H37Ra and H37Rv by allelic exchange. The *eis* gene was replaced by homologous recombination with a mutated *eis* allele containing a hygromycin resistance gene. During the selection process, 37 °C was accidentally used instead of 32 °C as the permissive temperature, so colonies grew faster. Similar to the findings of Pellicic *et al.*, the frequency of desired allelic exchange events was extremely high. Southern and Western analysis confirmed that after ts-*sacB* double selection, all fifteen selected potential allelic exchange *eis* mutants had wild-type *eis* replaced by *eis::hyg*. No single cross-over or random integration background was observed (data not show). This demonstrates that the ts-*sacB* vector is a valid system for allelic exchange mutagenesis in *M. tuberculosis*. The *eis* mutant was also complemented with a single copy of wild-type *eis* gene by using a vector that integrates at the *M. tuberculosis* chromosomal *attB* sites. Western blot analysis showed that the reintroduction of *eis* restores the production of the Eis protein to a level the same as that of wild-type, indicating a successful complementation.

Studies were done to compare the ability of the *M. tuberculosis eis* mutant to infect and proliferate within U-937 macrophages to that of its parental and complemented strains in an *in vitro* infection model. The *in vitro* studies revealed that the *eis* gene seems to not play a detectable role in intracellular survival and replication of both

H37Ra and H37Rv in U-937 cells, since no differences between the *eis* mutant and the parental strain were observed (Figure 23 and 24).

Although this result was discouraging, it does not completely exclude the possibility that the *eis* gene may still be involved in intracellular survival of *M. tuberculosis* in macrophages. First, the difference in phenotype might not be detected. There is a great number of null mutations that do not yield phenotypes, most likely because the range of conditions tested is narrow (201). Although we have observed that there was no difference between the intracellular growth of the *eis* mutant and that of the wild type within the human macrophage-like cell line U-937, our study did not address the survival of the *eis* mutant in either normal or activated primary human macrophages. It still remains possible that an intracellular survival defect of the *eis* mutant can be uncovered in INF- γ activated-macrophages or human monocytes. Several studies have found that INF- γ significantly enhances both phagosome maturation and the mycobacterial inhibitory capacity of macrophages (266, 305). Human peripheral monocytes that are more directly relevant to human tuberculosis compared with macrophage cell lines also should be tested to determine if *eis* prolongs the survival of virulent *M. tuberculosis* H37Rv in human peripheral monocytes (108).

It has been well known that cell-mediated immunity plays a critical role in the host defence against tuberculosis (223). In addition to macrophages, various T cell subsets and a variety of different cytokines are also involved in immunity to tuberculosis. Using macrophage culture as a model system in the infection assay would not reflect the interactions among the cell types *in vivo* which are important in the development of

immune responses against *M. tuberculosis*. Thus, conditions for multiplication and survival of organisms in macrophages *in vitro* may be different from those *in vivo*. Therefore, even with a lack of an cell-culture phenotype, deletion of the *eis* gene may still result in phenotypic differences between the *eis* mutant and its parental strain which may only be evident in an animal model.

Several reports have shown the discrepancy of mycobacterial virulence between *in vitro* and *in vivo* studies. Wedlock *et al.* have shown that a catalase-negative *M. bovis* strain, Wag405, could survive and grow as well as wild-type *M. bovis* within LPS and INF- γ -activated macrophages. Yet in comparison to the wild-type parental strain, Wag405 is avirulent in both guinea pigs and cattle (310). To investigate whether the *sigF* gene plays a role in persistence of *M. tuberculosis in vivo*, Bishai and his colleagues (55) inactivated the gene by allelic exchange (55). They found that the *sigF* mutant and the wild-type *M. tuberculosis* showed no differences in short-term (7-day) intracellular growth within human monocytes. In contrast, in long-term *in vivo* mouse survival studies, the loss of *sigF* reduced the virulence of *M. tuberculosis* for mice.

There also remains the possibility that *eis* does play a role in intracellular growth, but inactivation of the *eis* gene is compensated for by other redundant/backup mechanisms, and it was not to be detected phenotypically in the U-937 assay. Macrophages have developed numerous antimicrobial mechanisms such as low pH, degradative enzymes, and production of ROI and RNI products to kill intracellular bacteria (107). An abundance of overlapping stress response regulatory pathways has been observed in tubercle bacilli to avoid these antimycobacterial activities. It has been

proposed that *M. tuberculosis* antioxidant enzymes such as catalase (KatG), superoxide dismutase (331, 332), and alkylhydroperoxide reductase (AhpC) (53) can inactivate toxic oxygen molecules produced by macrophages. Numerous RNI-resistance genes have been identified in *S. typhimurium*: the *dpp* gene may interfere with production or uptake of RNI by macrophages (104); the *oxyR*, *ahpC*, *hmp*, and *zwf* genes may be involved in conversion of RNI to less toxic forms (214); *recBC* may be involved in repair of RNI-dependent lesions (274). In *M. tuberculosis*, a few RNI-resistance genes such as *ahpC*, *noxR1* (95), and *noxR3* (260) have also been identified. Although many of these antimycobactericidal activities are indispensable for intracellular survival, some of them might be redundant, and therefore could be compensated for by other similar activities. Recently, Stewart *et al.* showed that the deletion of the putative antioxidant *noxR1* gene did not alter the virulence of *M. tuberculosis* H37Rv, suggesting that the *noxR1* mutant could be compensated for by other antioxidant genes such as *noxR3* and *aphC* (286). To elucidate the role of these activities, multiple-deletion constructs are needed. Uncovering the function of the Eis protein would help to identify functional homolog(s) of Eis in *M. tuberculosis*.

The last possibility is that *eis* is mutated or not functional in the virulence of *M. tuberculosis* H37Rv but has an active role in other clinical strains such as CDC1551. Comparative genomics has revealed strain variation between the virulent laboratory strain H37Rv and its avirulent derivative H37Ra, suggesting that multiple mutations at different chromosomal loci are responsible for the attenuation of the bacillus (43). The recently isolated virulent strain CDC1551 is not more virulent than H37Rv and other

clinical isolates, but it induces a more rapid host immune response (193). To investigate the strain variation in H37Rv and CDC 1551 at DNA sequence level, the genome of *M. tuberculosis* CDC1551 is presently being sequenced (<http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gmt>). From the initial comparisons of the H37Rv and CDC1551 genomes there appears to be differences between the DNA sequences (113), although how these differences may relate to potential genes in respect to virulence or infectivity is presently unclear. The fact that Eis is present, and expressed in *M. tuberculosis* strain H37Ra and H37Rv makes it unlikely that large deletions have occurred in the *eis* region. However, mutations such as in-frame shift or point mutation in the *eis* gene could result in a dysfunctional gene product in different strains. The variation of *katG* region in *M. tuberculosis* strain H37Rv and other clinical isolates has been reported (333). A point mutation (G-->T) in *katG* was found only in INH-resistant *M. tuberculosis* strains but not in INH-susceptible strains (63). Because of a paucity of knowledge concerning its mechanism of action, no functional analysis of Eis has been done. To solve the issue whether Eis plays a more important role in pathogenesis of clinical isolates, the *eis* gene will be inactivated in these clinical virulent strains, and the resulting mutants will be tested in both *in vitro* and *in vivo* systems for any phenotypic changes.

To elucidate whether the *eis* gene has a role in the pathogenesis of tuberculosis, further studies are being directed towards identifying the stage at which the *M. tuberculosis eis* gene is needed during the infection and whether the disease produced

by the *eis* mutant differs immunopathologically from that caused by wild-type *M. tuberculosis*.

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