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Mycobacterium tuberculosis Pathogenesis and Molecular Determinants of Virulence

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INTRODUCTION

Tuberculosis (TB), one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. New vaccines and drugs are needed to stem the worldwide epidemic of TB that kills two million people each year. To rationally develop new antitubercular agents, it is essential to study the genetics and physiology of *M. tuberculosis* and related mycobacteria. It is equally important to understand the *M. tuberculosis*-host interaction to learn how these bacteria circumvent host defenses and cause disease. The approaches described in this review identify *M. tuberculosis* genes that are or are potentially involved in virulence. In the future, some of these genes and the proteins they encode, as well as newly discovered ones, should provide new bacterial targets that can be used for creating vaccines and drugs as well as more selective diagnostic reagents. To help the reader better understand the context for these approaches, a summary of various aspects of TB is presented initially, including a history of the disease, its clinical manifestations, as well as host and bacterial responses during infection. Because of space considerations, this initial discussion must omit important areas and can only touch on the many topics covered. For more extensive background material, there are many excellent books and reviews (30, 100, 116, 245).

HISTORY OF TUBERCULOSIS

TB, as described in the next section, can present in various forms, including one that attacks bone and causes skeletal deformities. Hard tissues like bone can be preserved for thousands of years, allowing the almost certain identification of individuals with bone TB who died more than 4,000 years ago. The frequency of unearthed skeletons with apparent tubercular deformities in ancient Egypt suggests that the disease was common among that population. The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark, and countries in the Middle East also indicates that TB was found throughout the world up to 4,000 years ago. The origin of *M. tuberculosis*, the causative agent of TB, has been the subject of much recent investigation, and it is thought that the bacteria in the genus *Mycobacterium*, like other actimomycetes, were initially found in soil and that some species evolved to live in mammals. The domestication of cattle, thought to have occurred between 10,000 and 25,000 years ago, would have allowed the passage of a mycobacterial pathogen from domesticated livestock to humans, and in this adaptation to a new

host, the bacterium would have evolved to the closely related *M. tuberculosis.* Specifically, it has been hypothesized that *M. bovis*, which causes a TB-like disease in cattle, was the hypothetical evolutionary precursor of *M. tuberculosis* (274). This hypothesis is now considered doubtful in the light of new data, since it was formulated before the genomes in the *M. tuberculosis* complex, including the human and animal pathogens *M. africanum*, *M. microti*, and *M. canetti*, as well as *M. tuberculosis* and *M. bovis*, were characterized by DNA sequencing and related methods. These studies have shown a greater than 99.9% similarity of DNA sequence among the members of the *M. tuberculosis* complex (38), but the existence of rare synonymous single-nucleotide polymorphisms (sSNP) allows discrimination between these closely related bacteria. sSNP analyses suggest that *M. bovis* evolved at the same time as *M. tuberculosis* (273), and a study of the distribution of deletions and insertions in the genomes of the *M. tuberculosis* complex provides strong evidence for the independent evolution of both *M. tuberculosis* and *M. bovis* from another precursor species, possibly related to *M. canetti* (38).

In recorded history, Assyrian clay tablets describe patients coughing blood in the seventh century B.C., and Hippocrates (fifth century B.C.) writes of patients with consumption (the Greek term is phthisis), i.e., wasting away associated with chest pain and coughing, frequently with blood in the sputum. By this time, the frequency of descriptions of patients with TB-like symptoms indicates that the disease was already well entrenched. It is thought that TB may have been introduced into these regions by the migration of Indo-European cattle herders who were carrying it by virtue of their exposure to cattle infected with the tubercle bacillus. Analysis of various human phenotypic traits, like lactose tolerance, that are associated with the raising of cattle and selection for the ability to utilize milk, as well as the resulting exposure to *M. tuberculosis*, has also suggested that Indo-Europeans spread the disease to Europe and Asia during their migrations into these regions (118).

Europe, with its population explosion in the second millennium A.D. and the growth of large urban centers, become the epicenter for many TB epidemics starting in the 16th and 17th centuries. This disease peaked in Europe in the first half of the 19th century, and it is estimated that one-quarter Europeans died of TB. In one study in a Paris hospital at this time, 250 of 696 cadavers examined showed that the individuals had died of this disease (77). In the last half of the 19th century, mortality due to TB decreased, largely due to improved sanitation and housing, of which the best-known example is the urban renewal of Paris in the 1850s, initiated and directed by Baron Georges Haussmann. Of course, the motivation for this massive project was not only public health concerns but also political considerations, since the wide, straight boulevards of the rebuilt Right Bank allowed better control of the increasingly radicalized working class by Louis Bonaparte's troops (51). It has also been postulated that natural selection of humans resistant to TB may have played a major role in the 19th-century decrease in the incidence of this disease, but the decline has been too rapid to be explained by these changes (165).

European immigrants to the New World brought the disease with them, and while the mortality rate never reached the levels found in Europe, large urban centers like Boston and New York had TB death rates of 6 to 7 per 1,000 in 1800, declining to 4 per 1,000 in 1860 to 1870 (62). Presumably public health measures also played a role in these declining mortality rates.

TB morbidity and mortality rates due to TB steadily dropped during the 20th century in the developed world, aided by better public health practices and widespread use of the *M. bovis* BCG vaccine (discussed below), as well as the development of antibiotics in the 1950s. This downward trend ended and the numbers of new cases started increasing in the mid-1980s. The major causes of this were increased homelessness and poverty in the developed world and the emergence of AIDS, with its destruction of the cell-mediated immune response in coinfected persons. Only by massive expenditures of funds and human resources, mainly by directly monitored antibiotic delivery, has this "miniepidemic" of new TB cases been reversed in Europe and the United States (99).

However, the underdeveloped world is still suffering from TB, as shown by the following statistics. The incidence of TB ranges from less than 10 per 100,000 in North America to 100 to 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa. There is one death from TB every 15 s (over two million per year), and eight million people develop TB every year. Without treatment, up to 60% of people with the disease will die (152). Essentially all these cases are in the Third World (318a), reflecting the poverty and the lack of healthy living conditions and adequate medical care (301). This global crisis is compounded by the emergence of multidrug resistance in countries like the former Soviet Union, South Africa, and India, where some antibiotics are available but are of inferior quality or are not used for a sufficient time to control the disease according to recommended regimens (140, 203).

Throughout the centuries, doctors and scientists have described TB in its many forms and sought to understand the origins of the disease, in order to use this information for better diagnoses, prevention, and cures. Hippocrates thought the disease was largely inherited, while Aristotle (4th century B.C.) stressed its contagious nature, as did Galen, greatest of Roman physicians, in the 2nd century A.D. This opposing view of the origins of TB reemerged in the second half of 17th century, where Italian physicians, continuing Galen's ideas and influencing countries in the Mediterranean basin, still maintained that TB was contagious. Conversely, doctors and savants in Northern countries favored constitutional or hereditary causes of this disease. Reflecting the empiricism of medical authorities of the time like Paracelsus of Switzerland, it was believed that the Southern theory of contagion was not rigorously proven scientifically and did not explain why some people in urban settings did not get TB even where there was a high incidence of the disease (118). This philosophic difference, which can be paraphrased as the well-known nature-versus-nurture conundrum, came to its high point in the 19th century. In 1865, Jean-Antoine Villemin, a French military physician, reported that he had been able to give TB to laboratory rabbits by inoculating them with tuberculous tissue from a cadaver. This report was immediately assailed by the French medical establishment, notably Herman Pidoux, who strongly maintained that there had to be more "modern" and more social solutions to the problem of TB, which he and others felt arose in the poorer (working) classes from external causes like mal-

nutrition, poor sanitation, and overwork. The report by Robert Koch 17 years later (155), which conclusively showed that TB was indeed caused by a bacterium discredited many of Pidoux's arguments. However, belief in the societal causes of TB still continued into the early 20th century as the revolutionary syndicalist movement in France, in their struggle for an 8-h working day, used TB as an example of a disease that was caused by overwork and malnutrition. Contemporary exponents of this view tried to discredit Koch's conclusive experiments, using arguments similar to those of Northern European doctors of the 17th century and Pidoux and his colleagues (17).

Starting with Edward Trudeau's work in the late 19th and the early 20th centuries, the apparent dichotomy in explaining the etiology of tuberculosis was resolved. In a classic experiment, which by today's standards might be considered statistically limited, he showed that TB could be induced in rabbits with a purified culture of virulent *M. tuberculosis* but that the environmental conditions in which the animals were maintained greatly influenced the course of the disease (290). In this study, five *M. tuberculosis*-infected rabbits were kept in a crowded, dark cage with minimal food. Of these, four died of TB within 3 months, and one became severely ill with the disease. When five similarly infected animals were allowed to live outdoors on a small island with additional food, one rabbit died within a month of infection but the other four were still alive after 6 months, with no sign of the disease. The control series, i.e., five uninfected rabbits confined to a dark, crowded cage with little food, became malnourished and clearly unhappy but did not get TB (290). This simple experiment gave scientific validity to the treatment of TB (fresh air and ample food) that was the basis of the TB sanitarium movement started by European physicians in the mid-1800s and that was also used by Trudeau in his Saranac Lake TB treatment center that opened in 1884. The history of research and treatment of TB at the Trudeau Institute has been described in a fascinating and informative review (57).

Thus, TB is caused by a bacterium, but environmental factors play a major role, an idea that Rene Dubos clearly rearticulated 50 years ago (77). To Dubos, purely medical solutions alone would not work to cure and prevent TB. Unfortunately, the events of the last half of the 20th century have shown how prescient he was. The antibiotic era, begun by the discovery of streptomycin by Schatz and Waksman in the 1940s and its use to treat TB and followed by the introduction of many other antibiotics like isoniazid, rifampin, and pyrazinamide that are useful against TB, has not eliminated the disease (248). Likewise, the widespread use of BCG, an attenuated vaccine strain produced by the sequential passage of a virulent *M. bovis strain* by Calmette and Guerin in Paris in the 1920s, has not lowered the incidence of TB in recent years (4), and there is more TB today than ever before (301). Clearly, new vaccines and drugs are needed for TB control, and approaches discussed in this review are designed to help in this search. However, it is always important to remember Dubos' cautionary words, which stressed the social nature of TB.

CLINICAL MANIFESTATIONS

As discussed earlier in this review, pulmonary TB has been variously described as consumption and phthisis, both terms indicating the severe wasting and the coughing of blood associated with later stages of the disease. Pott's disease or spinal tuberculosis, marked by spinal deformity and other bone defects, was named after an 18th-century English physician, but Hippocrates thought there was a great similarity between this bone disease and pulmonary tuberculosis and possibly a common origin. Scrofula, or cervical lymphadenitis, was a common disease in the middle ages that presented with swelling of lymph nodes in the neck. It was also called "The King's Evil" because of the myth that it could be cured by the touch of a reigning monarch. Villemin (mentioned above) showed in the 1860s that scrofula and pulmonary TB had an identical cause. Tuberculosis also can develop in the central nervous system, in which case meningitis is the predominant form of the disease, and also in the urogenital tract, the digestive system, and cutaneously in the form named lupus vulgaris. The incidence of these various extrapulmonary forms of tuberculosis varies from country to country, such that on the average between 1964 and 1989, 20% of the 20,000 new cases of TB in the United States were extrapulmonary while 5 to 10% of the approximately seven million new cases each year in the developing countries were extrapulmonary (283). This distribution also can be affected by origin of the individuals within a country. In one study of TB patients in England, 20% of patients of European origin had extrapulmonary TB, of which lymph node, bone and joint, and genitourinary involvement accounted for almost 90%. Of patients whose origin was on the Indian subcontinent, 45% had extrapulmonary tuberculosis, and 60% of these sites of infection were in lymph nodes and in bones and joints (319). Autopsies of deceased human immunodeficiency virus (HIV)-negative TB patients in another study in New York City showed that 68% had extrapulmonary TB whose lesions were widely and randomly distributed throughout the body with no apparent predilection for a limited number of sites as noted in the English study (143).

In modern times, most TB infections are initiated by the respiratory route of exposure now that milk products are generally pasteurized, at least in the developed world. One study in 1978, prior to the AIDS epidemic, showed that 85% of new TB cases were pulmonary (132). Thus, the different forms of the disease discussed above usually arise from dissemination of the bacilli from infected lungs. TB in many cases follows a general pattern as described by Wallgren, who divided the progression and resolution of the disease into four stages (302). In the first stage, dating from 3 to 8 weeks after *M. tuberculosis* contained in inhaled aerosols becomes implanted in alveoli, the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary or Ghon complex. At this time, conversion to tuberculin reactivity occurs. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung; at this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis or miliary (disseminated) tuberculosis. Pleurisy or inflammation of the pleural surfaces can occur during the third stage, lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. It is thought that this condition is caused by either hematogenous dissemination or the release of bacteria into the pleural space from subpleural concentrations of bac-

teria in the lung. The free bacteria or their components are thought to interact with sensitized CD4 T lymphocytes that are attracted and then proliferate and release inflammatory cytokines (149). The last stage or resolution of the primary complex, where the disease does not progress, may take up to 3 years. In this stage, more slowly developing extrapulmonary lesions, e.g, those in bones and joints, frequently presenting as chronic back pain, can appear in some individuals. However, most humans who are infected with TB do not exhibit progression of the disease. One-third of exposed HIV-negative individuals become infected, and of this number 3 to 5% develop TB in the first year. An additional 3 to 5% of those infected develop TB later in their lives. It is thought that most adult TB in non-HIV-infected patients is caused by reactivation of preexisting infection (104). HIV-positive persons infected with *M. tuberculosis* have a 50% chance of developing reactivation (postprimary) TB at some time in their lives. These individuals and others who are immunosuppressed can also be newly infected with *M. tuberculosis* and in many cases show rapid progression to active disease (105). Adult TB, whether resulting from activation or new infection in HIV-infected patients, is almost always pulmonary and is associated with differing degrees of lung involvement and damage, notably necrosis, cavitation, and bleeding (143).

M. TUBERCULOSIS VIRULENCE AND THE DISEASE PROCESS

The previous section described the different stages of human TB at the level of the infected patient and the involved organ systems. This section summarizes events in infection from the cellular and molecular viewpoint of both the infecting bacterium and its host. An excellent review of host innate immunity and responses to *M. tuberculosis* infection has recently appeared in this journal (297), and to avoid repetition, some areas are mentioned only briefly.

Events in the Infectious Process

Early events. As discussed above, *M. tuberculosis* usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes. This cell type is found in greater numbers than macrophages in alveoli, and *M. tuberculosis* can infect and grow in these pneumocytes ex vivo (24, 190). In addition, dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages (286) and presumably play a key role in activating T cells with specific *M. tuberculosis* antigens (31, 114). Since dendritic cells are migratory, unlike differentiated macrophages (164), they also may play an important role in dissemination of *M. tuberculosis.* However, this discussion is limited to the much more extensively studied and better understood *M. tuberculosis*-macrophage interaction. The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors (254). Surfactant protein A, a glycoprotein found on alveolar surfaces, can enhance the binding and uptake of *M. tuberculosis* by upregulating mannose receptor activity (107). On the other hand, surfactant protein D, similarly located in alveolae, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (90), and it is proposed that this prevents *M. tuberculosis* interaction with mannose receptors on the macrophage cell surface. Cholesterol in cell plasma membranes is thought to be important for this process, since removal of this steroid from human neutrophils decreases the phagocytosis of *M. kansasii* (221) and similar depletion experiments prevented the entry of *M. bovis* BCG into mouse macrophages (106). The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (201), and this important interaction with bacterial components is discussed later in this review. On entry into a host macrophage, *M. tuberculosis* and other intracellar pathogens initially reside in an endocytic vacuoule called the phagosome. If the normal phagosomal maturation cycle occurs, i.e., phagosome-lysosome fusion, these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Reactive nitrogen intermediates (RNIs) produced by activated mouse macrophages are major elements in antimicrobial activity (197), and mice with mutations in the gene encoding the macrophage-localized cytokine-inducible nitric oxide synthase gene are more susceptible to various pathogens, including *Leishmania major* (311), *Listeria monocytogenes* (169), and *M. tuberculosis* (168). The *M. tuberculosis* result is consistent with the results of other experiments showing that RNIs are the most significant weapon against virulent mycobacteria in mouse macrophages (48, 50) and the observation that resistance to RNIs among various strains of *M. tuberculosis* correlates with virulence (48, 50, 202). The presence of RNIs in human macrophages and their potential role in disease has been the subject of controversy, but the alveolar macrophages of a majority of TB-infected patients exhibit iNOS activity (200).

Since most macrophage killing of bacteria occurs in the phagolysosome (89), intracellular pathogens have evolved many ways to avoid this hostile vacuolar microenvironment. *Listeria* and *Shigella* physically escape the phagosome and replicate in the cytoplasm (252), and *Legionella* inhibits phagosome-lysosome fusion (134). *Salmonella enterica* serovar Typhimurium phagosomes also are diverted from the normal endocytic pathway of phagosome-lysosoma fusion (42, 233), and this bacterium requires acidification of the phagosome to survive in macrophages (234). Pathogenic mycobacteria also inhibit phagosome-lysosome fusion (6, 98), but unlike the situation for *Salmonella*, the mycobacterial phagosome is not acidified (60). This is presumably due to the exclusion of proton ATPases from the mycobacterial phagosome (281), but it is not clear that the blocking of endosomal maturation is essential for *M. tuberculosis* survival in macrophages. Live *M. tuberculosis* cells can made to traffic to late endosomes by opsonization with polyclonal antibodies against *M. tuberculosis* H37Rv, which presumably directs bacterial binding to Fc receptors. However, this rerouting has no effect on bacterial growth in mouse peritoneal macrophages (6). On the other hand, a recent study in which human monocyte-derived macrophages (MDMs) were infected with *M. tuberculosis* Erdmann opsonized with a polyclonal antibody raised against the *M. tuberculosis* cell surface glycolipid lipoarabinomannan (LAM)

showed that this treatment causes 80% loss of bacteria as well as increased trafficking to late, more acidic endosomes (175). The different results in these two experiments have not been resolved but could be in part due to the source of the macrophages, the nature of the antibodies, and the bacterial strains used. An interesting finding in the latter work is that Ca^{2+} signaling is inhibited when *M. tuberculosis* enters human macrophages but not when killed *M. tuberculosis* or antibody-opsonized *M. tuberculosis* cells are phagocytosed (175). This effect was correlated with trafficking to late endosomes; i.e., elevated Ca^{2+} levels were associated with phagolysosome formation. Since Ca^{2+} can stimulate many host responses to infection, e.g., the respiratory burst as well as NO and cytokine production, preventing increases in Ca^{2+} levels would help *M. tuberculosis* avoid these host defense mechanisms. It has also been postulated that a selective advantage to *M. tuberculosis* of staying in an early endosome is that there would be less host immunosurveillance by $CD4^+$ T cells. In agreement with this idea, there is a decrease in the expression of major histocompatibility complex class II (MHC-I) proteins and in the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection (201). As discussed below, this effect seems to be induced by presence of the secreted or surface-exposed *M. tuberculosis* 19-kDa lipoprotein, which is thought to interact with TLR2 in the early phase of bacterial entry into macrophages (287). The mechanism by which virulent mycobacteria prevent phagosomal maturation is not known, but in the normal maturation of the mycobacterial phagosome there is a successive recruitment of Rab proteins, which are small GTPases involved in endosome trafficking; i.e., Rab5 associates with early endosomes, and Rab7 is found in later endosomes. The *M. tuberculosis* phagosome that does contain Rab5 does not recruit Rab7 (298). Also, TACO, a member of the coronin family of actin binding proteins, is preferentially recruited to the mycobacterial phagosome of infected murine macrophages, where it was reported to be retained in phagosomes containing live and not killed *M. bovis* BCG (91). However, a more recent study, in which phagosomes and other macrophage organelles were isolated, has shown that the association of coronin with phagosomes containing live *M. bovis* BCG in both murine and human macrophages is transient and is retained only on phagosomes containing clumped bacteria (257). These latter results suggest that coronin is not involved with the arrest in phagosome trafficking observed in *M. tuberculosis* infections of macrophages. It is also not known whether the exclusion of Rab7 and/or the decreased Ca^{2+} signaling discussed above is directly responsible for this block in phagosome maturation or is a secondary consequence.

Later events. The relative ease of working with tissue culture has provided many data on *M. tuberculosis* entrance and trafficking in the macrophage and on other responses of the infected cells, as discussed above, but there is much less information on how the bacterium survives and grows during later stages of infection in the lung. It is known that infected macrophages in the lung, through their production of chemokines, attract inactivated monocytes, lymphocytes, and neutrophils (297), none of which kill the bacteria very efficiently (89). Then, granulomatous focal lesions composed of macrophage-derived giant cells and lymphocytes begin to form. This process is generally an effective means of containing the spread of the bacteria. As cellular immunity develops, macrophages loaded with bacilli are killed, and this results in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes (63). Although *M. tuberculosis* bacilli are postulated to be unable to multiply within this caseous tissue due to its acidic pH, the low availability of oxygen, and the presence of toxic fatty acids, some organisms may remain dormant but alive for decades. The strength of the host cellular immune response determines whether an infection is arrested here or progresses to the next stages. This enclosed infection is referred to as latent or persistent TB and can persist throughout a person's life in an asymptomatic and nontransmissible state. In persons with efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by an unknown process and then serves as a rich medium in which the now revived bacteria can replicate in an uncontrolled manner. At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB). When this happens, the person becomes infectious and requires antibiotic therapy to survive (63).

Currently, there is little information concerning how *M. tuberculosis* responds to the environment of the lung, preventing the development of rational strategies for treating latent and chronic infections as well acute manifestations of the disease. Some experiments on *M. tuberculosis* "persistence" or "latency" have been performed, using two chronic-infection models in mice in which bacteria can be maintained in a steady state in the absence of disease or are actually not cultivatible (reviewed in reference 95). However, it is still not certain whether bacteria in the chronic-disease models are actually viable but nongrowing, which would reflect a true latent state, or whether they are growing and dying at the same rate. The fact that *M. tuberculosis* in a chronically infected mouse model is susceptible to isoniazid (INH) (238), a drug that is effective only against growing *M. tuberculosis* (308), provides evidence for the latter explanation, i.e., balanced growth and death. There is biochemical evidence that the intermediary metabolism of *M. tuberculosis* changes, during the course of chronic mouse infections, from an aerobic, carbohydrate-metabolizing mode to one that is more anaerobic and lipid utilizing (258, 259). It is only in recent years that the full significance of these apparent changes in intermediary metabolism for acute or chronic infection has become apparent, as discussed later in this review.

DEFINING *M. TUBERCULOSIS* VIRULENCE

What makes *M. tuberculosis* virulent? Unfortunately, there is no simple answer yet, despite the knowledge obtained in the last 100 or more years. *M. tuberculosis* does not have classical virulence factors like those which are the major causes of diseases due to other bacterial pathogens, e.g., toxins produced

by *Corynebacterium diphtheriae*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, and *Vibrio cholerae.* The goal of much of the research discussed in this review is to help answer this question, i.e., to define *M. tuberculosis* virulence by finding the factors that are important for the progression of TB. While there is limited knowledge of how *M. tuberculosis* causes disease, its virulence can be measured. This quantitative view can then used to ascertain the effects of modifying the bacterium on the disease process. The standard terms "mortality" and "morbidity" are used for a description of *M. tuberculosis* virulence and can be defined in the following ways: mortality is the percentage of infected animals that die and is also measured as the time taken for an animal to die after being infected. Another important parameter that is usually associated with virulence is bacterial load or burden, i.e., numbers of bacteria found in the infected host after the initial infection. This information allows a comparison of the fitness of different bacterial strains to survive host responses during an infection. In addition, *M. tuberculosis* virulence mutants that have lower bacterial loads during animal infections exhibit different growth curves during this process; in one publication, they were grouped into various classes: *sgiv* (for "severe growth in vivo") (i.e., mutants that do not replicate at all but either are cleared rapidly or can persist with no increase in cell numbers), *giv* (for "growth in vivo") (i.e., mutants that grow initially but at lower rates than the wild type), and *per* (for "persistence genes") (i.e., those growing normally at earlier stages but declining in numbers at the onset of cell-mediated immunity) (109). This classification of mutations, especially when more are obtained, will be useful for understanding how the stages of the infectious process are controlled by different bacterial genes. In this review, to conform to standard genetic nomenclature, *M. tuberculosis* mutants that show attenuated growth in mice are classified with the same terminology but as phenotypes, i.e., SGIV, GIV, and PER. Morbidity, as measured by histopathology analyses, is important to characterize another class of *M. tuberculosis* mutants affecting virulence, i.e., those that do not affect the bacterial load. An example of one of these is the *M. tuberculosis sigH* mutant, which shows normal *M. tuberculosis* growth and survival in macrophages and mice (151, 181) but whose histopathology in infected mouse lungs is much lower than that caused by wild-type *M. tuberculosis* (151).

To better measure morbidity and mortality caused by *M. tuberculosis* it is important to understand the pathogenesis associated with TB. Uncontrolled *M. tuberculosis* growth in its human host, given the usual site of the infection, is associated with extensive lung damage that ultimately causes death by suffocation due to insufficient oxygen. This anoxia is caused the obliteration of lung parenchymal cells involved in oxygen uptake as well as obstruction of bronchiolar passages by granulomatous growths and by blood released during the rupture of liquefied granulomas in adjacent lung tissue (104). Other untreated forms of tuberculosis such as tubercular meningitis, which occurs in the meningial membranes of the brain, can result in death because of inflammation in brain tissue and the resulting hydrocephalus and seizures. Tuberculomas, another form of TB in the brain, are large structures formed by by the enlargement of brain granulomas, also due to inflammatory responses, and they are also associated with seizures (329). Inflammatory responses are also thought to play a role in other extrapulmonary manifestations of TB, e.g., in bone (189).

Inflammation is a keyword here, since the growth of *M. tuberculosis* elicits inflammatory host responses that are necessary to control infections but can also cause extensive tissue damage. Among the cellular agents involved in tissue destruction are various proteases like cathepsin D (196) that are also thought to be major factors in the liquefaction of granulomas (58). In addition, *M. tuberculosis* uptake can cause apoptosis of macrophages (153, 157), and this could play a role in adjacent tissue damage. A key cytokine in the inflammatory or Th1 response of the cellular immune system is tumor necrosis factor α (TNF-α), which is necessary to control infection. Mice that are unable to produce or respond to TNF-α cannot form granulomas to restrict bacterial dissemination (260). However, when this cytokine is present in large amounts during an aerosol model of mouse infection, severe lung inflammation and early death occurs (21). TNF-α is a major determinant of disease in a rabbit model of TB meningitis, since there is a direct correlation between the extent of disease caused by several *M. bovis* and *M. tuberculosis* strains and levels of this cytokine in the cerebrospinal fluid (291). However, data from analyses of cytokine responses and virulence in mice infected with various *M. tuberculosis* strains indicate that there are factors additional to TNF-α in TB progression. *M. tuberculosis* CDC1551 is a clinical strain that was originally thought to be highly virulent (142); it has more recently been shown that CDC1551 induces levels of cytokines, including TNF-α, that are higher than those induced by other *M. tuberculosis* strains in mice. However, it is not more virulent than the other strains, as defined by bacterial load and mortality (177). Similar results were obtained when the virulence of CDC1551 and H37Rv were compared in a rabbit model of infection (29). Also, another study compared the ability of two clinical *M. tuberculosis* strains, HN878 and NHN5, to cause disease and to elicit a cytokine response in a mouse model. HN878, the more virulent of the two as determined by mortality measurements, induced smaller amounts of TNF-α and other inflammatory cytokines than NHN5 did (178). Interestingly, HN878 induced higher levels of alpha interferon (IFN-α) and Th2 cytokines like interleukin-4 (IL-4).

It has also been reported that the apoptosis that is sometimes observed when *M. tuberculosis* infects macrophages is dependent on TNF-α and that more virulent *M. tuberculosis* strains cause less apoptosis (10). The above experiments illustrate the complexity of the immune system and its effectors, since their results are not consistent with a simple direct relationship between the levels of one or a few cytokines such as TNF-α and the progression of the disease in clinical or model settings. Clearly, an optimal balance of these immunomodulators is crucial. Despite the different results, which make it difficult to interpret the data in these studies, they are of value because they show that some *M. tuberculosis* strains are more virulent than others in animal models (78).

Models for Measuring *M. tuberculosis* Virulence

Animal models. *M. tuberculosis* virulence is studied both in tissue culture, mainly using macrophages and more recently dendritic cells (31, 126, 145) and pneumocytes (24, 28), and in

animal models. While tissue culture models are easier to work with and give faster results, they are limited to the early stages of infection. Thus, the animal models are ultimately better because all the stages of TB can be studied. The choice of animal models for virulence studies is important, and the three major models, mice, guinea pigs, and rabbits, each have their advantages and disadvantages. Mice are the most frequently used in vivo models because of their well-studied genetics (the existence of inbred strains, including some that have mutations in the immune system) the availability of reagents to measure cytokine levels, and their low costs of maintenance relative to other animal models (205). Another big advantage to mouse experiments is that there are inbred strains that show widely different levels of resistance to *M. tuberculosis* and other pathogens, which allows the mapping of pertinent loci. In one such study that analyzed the offspring of a mating between the relatively sensitive C3H mouse strain and the resistant B6 strain, a locus, *sst1*, was mapped that had a major effect on susceptibility to tuberculosis (156). However, it was shown in these experiments that resistance or susceptibility to *M. tuberculosis* is a complex trait and that other, unidentified loci must also play a role. In an ambitious and significant study, a set of recombinant congenic mouse strains that were made by mating strains A/J and C57/BL6 is now being used to chromosomally map loci conferring resistance or sensitivity to bacterial infections (97). Hopefully, these mouse strains will be used in the near future to map the other loci determining host resistance to *M. tuberculosis* infections. The progression of TB in mice is unlike that in humans, in that the granulomas formed are not as distinct, but the fact that mice are generally not as sensitive to the disease as other animal models and can become chronically infected is more like the human situation. Guinea pigs are very sensitive to *M. tuberculosis* infection, and the stages of the disease, including early stages of granuoma formation, in this animal are similar to those in humans (205). The disadvantages are the lack of inbred strains and reagents as well as the high maintenance costs. The rabbit model has one big advantage over the other animal system in that the lung granulomas formed during the disease show the same progression of stages, i.e., caseation, liquefaction, and cavitation, as observed in advanced cases of human TB (58). The disadvantages of rabbits are similar to those of guinea pigs, but their upkeep is even more expensive.

Macrophages. Since *M. tuberculosis* is an intracellular pathogen and infects macrophages primarily, these phagocytic cells are also used to analyze the virulence of *M. tuberculosis* strains and mutants. These ex vivo studies serve as a model for the early stages of infection, which involve the phagocytosis of *M. tuberculosis* by resident macrophages in the lung alveoli. Since human alveolar macrophages are difficult to obtain, model macrophage systems are generally used. These can be from mice or humans and can be primary cultures or immortalized cell lines. Macrophages from other animals are not as useful because of the lack of suitable reagents. Mouse macrophages can be primary, isolated from bone marrow, from lung alveoli by bronchial lavage, and from peritoneal exudates after injection of thioglycolic acid into the peritoneal cavities of mice. Primary macrophages are natural (not immortalized) and are more representative of the actual in vivo situation, but they are usually harder to obtain and are more variable, especially macrophages from human donors. There are many mouse macrophage cell lines available, including the widely used J774 line and MH-S cells, the latter being an immortalized alveolar macrophage cell line whose behavior is very similar to that of primary mouse alveolar macrophages (191). Since the mouse is the most widely used animal model, there are advantages to using macrophages from this mammal, including reagent availability, as discussed above. In addition, the use of primary mouse cells allows the preparation of macrophages from animals with defined mutations so that the effect of specific host factors on interactions with *M. tuberculosis* at the macrophage level can be tested (83). Activation of primary mouse macrophages or macrophage cell lines by the addition of IFN-γ and lipopolysaccharide, which induces the levels of the iNOS enzyme needed to form NO, is necessary to observe the *M. tuberculosis*-killing activity of these cells (50).

Human macrophages are also widely used, and a big advantage of these studies is that the early stages of human disease can be studied. Human macrophages used in these experiments are primary cultures derived from peripheral blood monocytes (MDMs) that are allowed to differentiate into macrophages, as well as the more difficult-to-obtain alveolar macrophages derived by branchiolar lavage. Transformed monocytic cell lines like THP-1, which can be differentiated into macrophage-like phagocytic cells by the addition of phorbol esters (292), are frequently used, as are similar immortalized monocytic cell lines like U937 (310). Studies have shown that differentiated THP-1 macrophages are quite similar to human MDMs in their response to *M. tuberculosis* infection (279). In addition, many reagents are available to study human host responses to *M. tuberculosis* infection. Human macrophages from TB-negative donors or cell lines do not kill *M. tuberculosis* well, presumably because they do not produce NO (69). This is an advantage if one is testing the survival of bacterial mutants, since the wild-type *M. tuberculosis* cells usually grow well and mutant growth phenotypes can be more accurately compared than in activated mouse macrophages (182).

There are certain caveats in using macrophages for virulence studies, however. Some *M. tuberculosis* mutants do not exhibit an attenuated phenotype in macrophages yet are defective for growth in mice and/or cause fewer histopathologic changes, as discussed later in this review (74, 275). Thus, genetic selections or screens for *M. tuberculosis* virulence phenotypes that use macrophages alone may miss some attenuated *M. tuberculosis* mutants. Another problem is that macrophages isolated from different organs of the same animal may respond differently to *M. tuberculosis* infection. A dramatic example of these tissue differences comes from a recent comparative study of the interactions between *M. tuberculosis* H37Rv and primary macrophages isolated from the sensitive I/St and resistant A/S mouse strains (174). In these experiments, macrophages isolated from lungs of the sensitive mice were killed more readily by *M. tuberculosis* and permitted better bacterial growth than did their counterparts from the resistant strain. On the other hand, peritoneal macrophages from the sensitive strain were more resistant to *M. tuberculosis* killing and restricted bacterial growth more than did the resistant peritoneal macrophages isolated from the resistant mice.

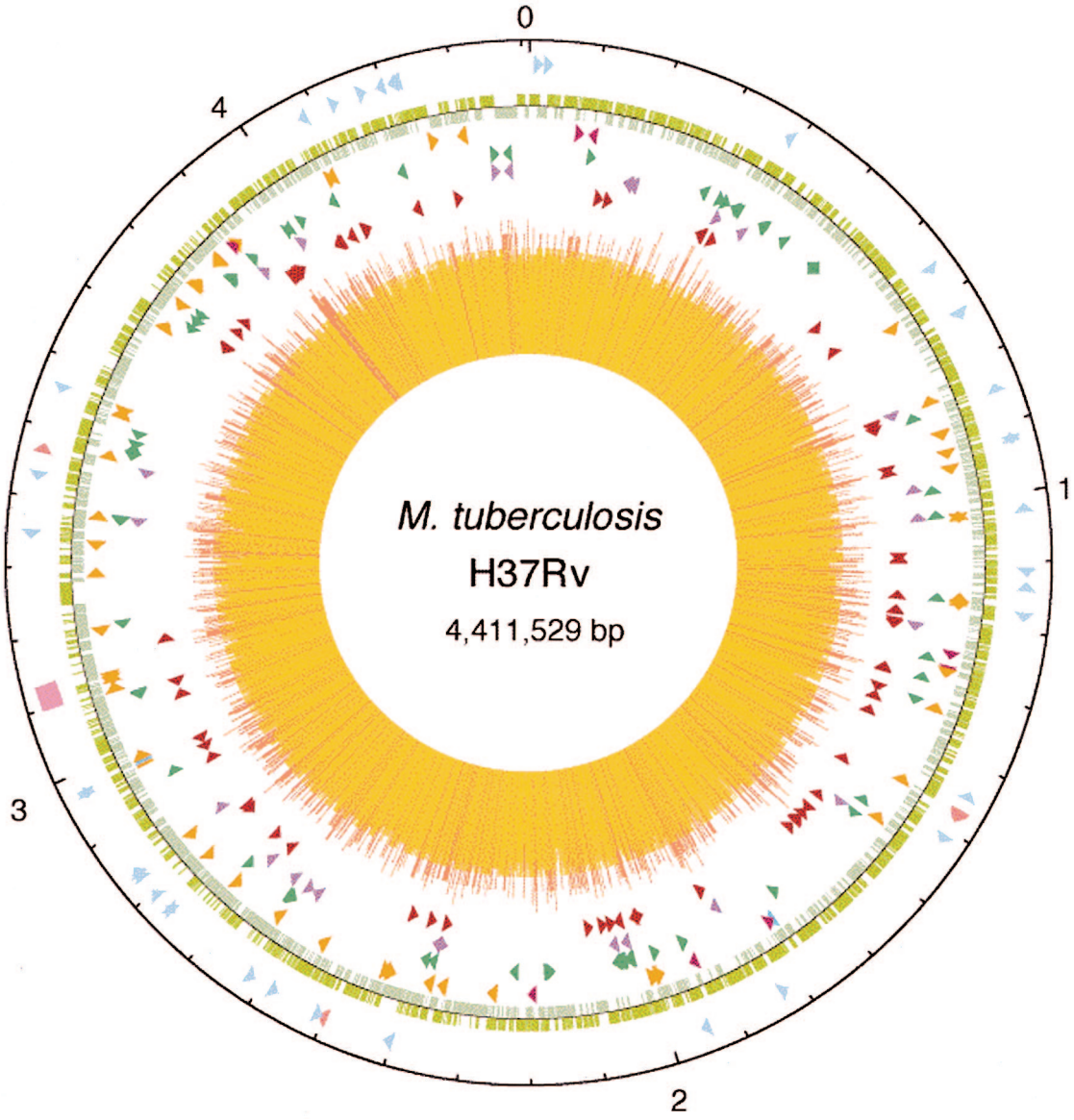

FIG. 1. Circular map of the chromosome of *M. tuberculosis* H37Rv. The outer circle shows the scale in megabases, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, and others are pink) and the direct-repeat region (pink cube); the second ring shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (center) represents the G+C content, with <65% G+C in yellow and >65% G+C in red. The figure was generated with software from DNASTAR. Reprinted from reference 53 with permission from the senior author and the publisher.

GENETICS OF *M. TUBERCULOSIS*

Until quite recently, the genetics of *M. tuberculosis* was a neglected subject because of difficulties in working with the organism and lack of suitable tools. A review published as recently as 1994 stated that this field ". . . is still in its infancy" (55), but the study of mycobacterial genetics has blossomed in recent years, as demonstrated by the publication an entire book dedicated to this topic (122). This is due to the development of many genetic methods, mainly by the Gicquel and Jacobs laboratories (109, 218), and to the DNA sequencing and annotation of the *M. tuberculosis* H37Rv genome (53) and those of related mycobacteria that have been or are currently being completed by The Institute for Genomic Research (94) and by the Sanger Center-Pasteur Institute consortium.

Description of the *M. tuberculosis* Genome

The *M. tuberculosis* H37Rv genome consists of 4.4×10^6 bp and contains approximately 4,000 genes (Fig. 1) (53). Annotation of the *M. tuberculosis* genome shows that this bacterium

TABLE 1. General classification of *M. tuberculosis* genes

Function	No. of genes	% of total	% of Total coding capacity
Lipid metabolism	225	5.7	9.3
Information pathways	207	5.2	6.1
Cell wall and cell processes	517	13.0	15.5
Stable RNAs	50	1.3	0.2
IS elements and bacteriophages	137	3.4	2.5
PE and PPE proteins	167	4.2	7.1
Intermediary metabolism and respiration	877	22.0	24.6
Regulatory proteins	188	4.7	4.0
Virulence, detoxification and adaptation	91	2.3	2.4
Conserved hypothetical function	911	22.9	18.4
Proteins of unknown function	607	15.3	9.9

has some unique features. Over 200 genes are annotated as encoding enzymes for the metabolism of fatty acids, comprising 6% of the total (Table 1). Among these are approximately 100 that are predicted to function in the β-oxidation of fatty acids, while *E. coli* only has 50 enzymes involved in fatty acid metabolism. The distantly related actinomycete *Streptomyces coelicolor* has a total of 115, corresponding to a little more than 1% of the proteins, of which 59 are annotated as being involved in fatty acid degradation (23). This large number of *M. tuberculosis* enzymes that putatively use fatty acids may be related to the ability of this pathogen to grow in the tissues of the infected host, where fatty acids maybe the major carbon source. This important aspect of *M. tuberculosis* physiology during infection is described later in this review.

Another unusual feature of the *M. tuberculosis* genome is the presence of the unrelated PE and PPE families of acidic, glycine-rich proteins. The names come from the Pro-Glu (PE) and Pro-Pro-Glu (PPE) sequences found in the two conserved N-terminal regions in each of these protein families that are approximately 110 and 180 amino acids long, respectively. The 172 genes, 104 of the PE class and 68 of the PPE variety, comprise over 4% of the genes in *M. tuberculosis*, and similar levels of abundance are noted in other members of the *M. tuberculosis* complex for which sequence data are available (39). PE and PPE genes are not unique to members of the *M. tuberculosis* complex, since *M. leprae* has 26 genes for these two families. Nineteen of these are pseudogenes, reflecting the extensive physical and genetic downsizing of the *M. leprae* genome by deletion and mutation during the evolution of this obligate parasite (54). *M. marimum*, a pathogenic mycobacterium that infects frogs and fish and causes a TB-like disease, has some PE genes that are involved in virulence (231). These proteins are not restricted to pathogenic mycobacteria, since *M. smegmatis* has some proteins of the PE-PGRS family (13). In *M. tuberculosis*, proteins encoded by the 104 PE genes can be further subdivided into three classes, containing 29 proteins with the PE region alone, 8 proteins in which the PE region is followed by unrelated C-terminal sequences, and 67 proteins that form the PE-PGRS subfamily. This group of proteins has the conserved PE domain followed by C-terminal extensions with multiple repeats of Gly-Gly-Ala or Gly-Gly-Asn that are in the PGRS (for "polymorphic GC-rich repetitive sequences") domains. The function of these large families of related proteins is unknown, but size variation has been observed in members of the PE-PGRS subfamily family in clinical TB strains, and many of these proteins have been localized in the cell wall and cell membrane (13). These data and the antigenicity of these proteins have led to the hypothesis that at least some of these proteins may be involved in antigenic variation of *M. tuberculosis* during infection (13). Significantly, many of the PE genes that encode proteins containing only the 110-amino-acid domain are closely followed by a gene encoding a PPE protein. In one case, a tandem PE-PPE pair, Rv2431c-Rv2430c, are coexpressed and can form a complex (M. Strong and D. Eisenberg, personal communication).

Otherwise, *M. tuberculosis* is not remarkable in its annotated content of most groups of genes and their products that are found in microorganisms. For example, among transcriptional regulatory proteins, *M. tuberculosis* has 13 sigma factors (proteins that confer transcriptional specificity on RNA polymerases), corresponding to 0.3% of the total genes and, 22 other regulatory proteins, including 13 two-component response regulators (usually transcriptional regulators that are activated by and serve to transduce environmental signals), corresponding to 0.6% of the total. These numbers are quite similar to the frequency of genes that encode these regulators in the genomes of *C. diphtheriae*, *Bacillus subtilis*, and *E. coli.* This is much lower than the corresponding numbers for the soil-dwelling, spore-forming *S. coelicolor*, which has 55 sigma factor genes, 0.7% of the total, and 74 response regulator genes, over 2% of the total (23). It has been postulated that the soil environment in which *Streptomyces* species are found has selected for the ability of these microorganisms to adapt to radically changing conditions. This would occur by by gene duplication and divergent evolution that would give rise to many transcriptional regulators, allowing appropriate bacterial responses to a changeable environment. In agreement with this idea of environmental selection, the number of predicted transport proteins encoded in the *S. coelicolor* genome is 614, corresponding to 8% of the total genes. The equivalent number in *M. tuberculosis* is 125 annotated genes for transport functions, corresponding to 3% of the total. Circumstantial evidence for this idea of gene duplication is provided by the fact that the *S. coelicolor* genome is over 8.5×10^6 bp, twice as big as that of *M. tuberculosis* and encoding twice as many proteins (23). There must be additional reasons for these dramatic differences in the *Streptomyces* genome compared to other eukaryotes. *B. subtilis* is also a spore-forming soil dweller, but its 4×10^6-bp genome has levels of regulatory and transporter proteins that are similar to bacteria other than *Streptomyces.*

Methods of Genetic Analysis in Mycobacteria

The complete sequence and annotation of the *M. tuberculosis* genome has allowed many new genetic approaches to studies of the physiology and pathogenicity of this organism, but much important work was done in this area before the genome-sequencing project was initiated. These pregenome approaches dealt largely with developing methods for creating mutations in specific genes. The choice of which genes to use and ultimately inactivate in order to study virulence was frequently based on on the existence of naturally mutations occuring in normally virulent strains that affected pathogenicity,

e.g., *sigA* and *katG* (56, 317), or predictions of which genes should be important in some aspect of *M. tuberculosis* virulence and/or physiology by inference from studies of other bacterial pathogens, e.g., genes encoding sigma factors and iron acquisition regulators (267). The following discussion addresses methods used for genetic analysis of mycobacterial species. Their application to the identification and characterization of *M. tuberculosis* genes that play a role in virulence is discussed later in this review. There are some methods, such as bacterial conjugation (211) and generalized bacteriophage-mediated transduction (282), that have been used for genetic studies of *M. smegmatis* but are not discussed here because they have not yet been successfully applied to *M. tuberculosis.*

Initial genetic studies. Early studies on the creation of mutations in mycobacteria concentrated on the faster-growing nonpathogenic species because of the relative ease of working with these bacteria. There is no requirement for biosafety level 3 containment facilities, and the experiments are relatively rapid; e.g., *M. smegmatis* has a 3-h generation time while that of *M. tuberculosis* is 20 to 24 h. Several techniques were developed to inactivate genes in these bacteria, with the first reports of *M. smegmatis* and *M. bovis* BCG transformation being published in 1988 (268) and the subsequent development of a highly transformable *M. smegmatis* strain, MC^2155, being published in 1990 (269). These and more recent articles have been reviewed (187). While these methods had some success in investigating *M. tuberculosis*, the process was still difficult. In addition to its extremely slow growth, which makes it time-consuming to do the standard types of gene inactivation (it takes 3 weeks for a single *M. tuberculosis* cell to become a visible colony on solid media), this bacterium was thought to have lower rates of homologous recombination and higher rates of illegitimate recombination than other mycobacteria, which would complicate gene disruption by standard gene replacement techniques. In fact, earlier attempts to inactivate *M. tuberculosis* genes by allelic exchange resulted in illegitimate recombination (148, 316). The original observation that the *M. tuberculosis* RecA protein was synthesized with an intein (protein intron) that had to be spliced out (65) led to speculation that this process contributed to the reported low levels of homologous recombination in this species (65, 187). However, later results indicated that the *M. tuberculosis recA* gene is equally competent in restoring function to a *M. smegmatis recA* mutant as is the *M. smegmatis* inteinless gene, suggesting that the splicing out of inteins does not affect the capability of RecA for homologous recombination (101, 208). In addition, more recent experiments have shown that *M. tuberculosis* has similar rates of homologous recombination to those of the faster-growing nonpathogenic *M. smegmatis* (214).

Current genetic methods. Despite the problems mentioned in the previous section, several current techniques have been successful in inactivating *M. tuberculosis* genes. Gene disruption techniques in mycobacteria, as described below, can be divided into directed and global methods but generally require a selectable phenotype, usually resistance to an antibiotic. The most frequently used antibiotic resistance cassettes in mycobacteria are those conferring resistance to kanamycin, hygromycin, and streptomycin (47). These antibiotics are also useful for selection in *E. coli*, allowing most cloning procedures to take place in this organism with appropriate plasmid vectors. Selection for kanamycin resistance (Kan^r) is favored in many mycobacteria like *M. smegmatis* because of the generally low levels of spontaneous Kan^r mutations and the high stability and low cost of the antibiotic. However, kanamycin resistance is not a good selective marker in *M. tuberculosis* because there is a high spontaneous mutation rate that results from the presence of only one rRNA (*rrn*) cistron in which the 16S rRNA gene can undergo mutations to Kan^r at a significant level. *M. smegmatis* has two *rrn* cistrons, and mutations to Kan^r in one of these is masked by the dominance of the second, sensitive *rrn* cistron (251). The high background to Kan^r in *M. tuberculosis* can be avoided by introducing a second resistance marker, such as a streptomycin resistance cassette, into the plasmid construct and doing a double-antibiotic selection for kanamycin and the second antibiotic (streptomycin in this case) (244), but this modification requires a more complex cloning strategy. Hygromycin resistance is the preferred antibiotic selection in *M. tuberculosis* in spite of its cost, because the frequency of spontaneous mutation to this drug is very low.

(i) Directed gene disruption. Directed gene inactivation entails the insertion of an antibiotic resistance cassette in the middle of the gene of interest and then the transformation of this DNA into mycobacteria as a linear or circular molecule, using electroporation. The desired result is allelic replacement of the chromosomal gene by the mutated one. In members of the *M. tuberculosis* complex, directed gene disruptions have been made with long linear molecules, up to 40 kb (9), or shorter ones, in the range of 4 kb (1, 240). The use of single-stranded linear DNA increases allelic replacement by homologus recombination in *Streptomyces* (204), and this effect is also observed in gene inactivation experiments in *M. smegmatis* and in the *M. tuberculosis* complex (127). The advantage of the linear-DNA method is that cloning is relatively easy, especially when short DNA fragments are used. A disadvantage is that unique restriction enzyme sites are required unless one employs more elaborate manipulation of the DNA sequence of interest, and this problem is magnified as the DNA increases in length. This is true for linear DNAs and plasmid-based systems described below. One early way of avoiding this problem was by the use of transposition systems that function in *E. coli* to disrupt genes contained in large segments of mycobacterial DNA that were cloned into plasmids with *E. coli* replicons. Many transposition insertion events in several genes contained in a 7-kbp *M. smegmatis* chromosomal fragment were obtained in *E. coli* using transposon mγδ-200 (96). The desired disruption in the *ideR* gene contained in the *M. smegmatis* DNA fragment, determined by restriction enzyme digestion analyses, was then used to create an *M. smegmatis ideR* mutant gene by homologous recombination, using techniques described in the next paragraph (79). A newer transposon delivery system uses a cell-free approach to disrupt mycobacterial genes. A complex, called the transpososome, is made between the transposable element Tn *5* containing a selectable antibiotic resistance marker and its transposase, the enzyme responsible for the integration of the transposon into other DNAs. The transpososome is commercially available and has been used to make random mutations in cloned mycobacterial DNAs as well as intact mycobacteria after electroporation of the transpososome into recipient bacteria (70).

Most directed mutations in mycobacteria are performed with bifunctional or shuttle plasmid vectors that can be maintained in *E. coli*, and many are available for this use (47). Circular plasmid integration, using a vector that cannot replicate in the recipient or has a temperature-sensitive (*ts*) replicon (216), is the most widely used method for directed gene disruption by allelic replacement in mycobacteria (187). It usually entails a two-step process in which the plasmid containing the desired gene disrupted with an antibiotic resistance cassette integrates into the genome by a single crossover event (Campbell-type integration) at the region of homology, selecting for the antibiotic resistance cassette. This event forms a direct repeat at one of two positions relative to the antibiotic cassette. In a second crossover event, the plasmid backbone is excised by means of recombination at the other direct repeat than the one initially used, when the antibiotic selection is maintained, resulting in the desired gene disruption. This technique can be relatively efficient when coupled with a counterselection to facilitate plasmid elimination in the second step. Two methods have been used for counterselection; the first is resistance to streptomycin (Str^r), using wild-type *rpsL* in the plasmid vector and a mutated *rpsL* (Str^r) allele in the chromosome of the recipient (250). Since the sensitive *rpsL* allele is dominant, resistance to streptomycin is observed only when it is lost along with the plasmid backbone, leaving the unique *rpsl* (Str^r) chromosomal gene. The second counterselection is sucrose resistance when *sacB* encoding levansucrase is in the plasmid backbone. This enzyme converts sucrose to levans that are toxic to cells such as mycobacteria that lack a functional levanase that coverts levans to fructose and glucose (215). In both cases, the presence of the vector in the bacterial chromosome prevents bacterial growth on selective media, i.e., either streptomycin or sucrose. Of the two methods, the *sacB* selection is easier because one does not have make a specific Str^r recipient in which *rpsL*, but not *rrnA*, is mutated. Another advantage to the *sacB* sucrose counterselection is that it can be used to introduce silent or unmarked mutations into the chromosome of the recipient, dispensing with the need for any antibiotic resistance marker. This method was used with *M. smegmatis* (217) and later with *M. tuberculosis* (214) and is necessary for the development of live, attenuated *M. tuberculosis* vaccine strains, as discussed later in this review. The drawbacks of the two-step procedure are, again, the necessity for a unique restriction site in the gene of interest coupled with the time required for the process, i.e., approximately 3 to 5 months from the initial transformation to the verification, by DNA analysis, of the gene disruption in survivors of the counterselection. In addition, mutations that inactivate the plasmid-borne *rpsl* or *sacB* during the selection and counterselection procedure will be erroneously scored as desired events in which the plasmid has been excised from the chromosome. A way of avoiding these false-positive results is to introduce another easily screenable marker in the plasmid backbone that should also be eliminated with the vector. An Str^r cassette (244) and reporter fusions that give a visible plate phenotype have been used for this purpose, including fusions with *xylE* (327) and *lacZ* (209).

A simpler and faster one-step variation of single-crossover gene inactivation with circular DNA has been used to inactivate mycobacterial genes. This technique takes advantage of the property of internal gene fragments carried on a circular DNA (e.g., a nonreplicating plasmid) to disrupt the corresponding gene when the DNA integrates into the chromosome by a single Campbell-type event. It has been possible to disrupt the *M. smegmatis sodA* gene with DNA fragments as short as 180 bp by this method (80). These events can be unstable, however, since recombination at the direct repeats in the chromosome formed during the plasmid integration, which is the basis of the two-step plasmid procedure, can lead to excision of the plasmid, restoring the intact wild-type gene, in the absence of the selecting antibiotic.

The major problem with the above methods is they are for the most part inefficient since the frequency of introducing DNA into mycobacterial species, especially *M. tuberculosis*, by the standard electroporation technique is quite low, even with the utilization of modifications that increase transformation, e.g., raising the temperature at which the DNA is introduced into *M. tuberculosis* (304). A new method of directed gene inactivation, termed specialized transduction, using a *ts* bacteriophage delivery system has been described recently that bypasses these problems and is the current method of choice for directed mutation (108). A recent review by the recently deceased S. Bardarov, the inventor of the mycobacterial specialized-transduction system, is quite useful because it gives a detailed description of the method. This article also shows how the γδ resolvase system can be used to make silent mutations in mycobacteria, allowing the creation of multiply mutated bacterial strains (14). In the specialized-transduction system, the gene of interest that has been disrupted with an antibiotic resistance cassette is cloned into a plasmid containing the mycobacteriophage phAE87 genome with a *ts* replicon and also has a bacteriophage lambda-packaging site. This allows the formation of viable transducing bacteriophage particles by an in vitro packaging reaction that can be transduced into *E. coli.* Cosmid DNA is prepared in *E. coli* and is then transformed into *M. smegmatis* at 30°C, the pemissive temperature which allows the formation of infectious mycobacteriophage. *M. smegmatis* lysates with the bacteriophage construct are then used to infect *M. tuberculosis* at 37°C the nonpermissive temperature, with high enough multiplicities to transduce all of the bacteria. Transductants are then selected on antibiotic-containing media for events in which the *M. tuberculosis* gene has integrated into the chromosome. This technique is very efficient because essentially all of the recipient cells can be transduced and the selection is robust.

(ii) Global gene inactivation. The principle of global gene inactivation is the insertion of foreign DNA, usually a transposable element, into many sites in the bacterial genome, ideally on a completely random basis. These events require a selectable phenotype, generally an antibiotic resistance marker carried within the transposable element. Two groups have developed efficient transposition systems that produce integration events in the genomes of mycobacterial species, including *M. tuberculosis.* These systems use transposable elements carried by vectors that cannot replicate at temperatures above 39°C: in one case, a *ts* plasmid that carries Tn *1096* (216), and in the other, a *ts* bacteriophage, similar to the one described in the previous section, that carries Tn *5367* or Tn *5370* (15). The advantage of these systems is that one can start with a transformed or infected population of cells and easily obtain many

integrative events by passage at the restrictive temperature. Both systems have been used to identify *M. tuberculosis* virulence genes by using the signature-tagged mutagenesis (STM) method developed to clone *Salmonella* genes essential for bacterial survival during mouse infections (124). In this technique, uniquely tagged transposon *M. tuberculosis* mutants were made in broth cultures and were negatively screened by hybridization for those that did not survive during animal infections (46, 59).

A drawback of these systems when used with *M. tuberculosis* is that the transposition events are not completely random in that there seem to be "hot spots" of transposition integration, based on the genes found in initial STM screens, as discussed below (46, 59). Since the *M. tuberculosis* genome has approximately 4,000 open reading frames (ORFs), many events would be needed to saturate the genome and mutagenize every gene when using a nonrandom inactivating system. A transposition system has been developed more recently from the mariner transposable element *Himar-1* that overcomes some of these drawbacks. The mariner element recognition sequence is simply an A-T base pair and is be expected to be truly random in its integration, unlike Tn *1096* and Tn *5367*, which recognize much larger integration sites. The *Himar-1* system has been used to introduce transposition events in *M. smegmatis* (247), *M. bovis* BCG (253), and, more recently, *M. tuberculosis* H37Rv, where 100,000 transposon insertions have been obtained. DNA sequencing and other analyses indicate that over 2,600 *M. tuberculosis* genes have been inactivated thus far (E. Rubin, personal communication). STM tagging was not used to make these mariner transposon mutations, and so there is no simple global screening method for virulence genes as there are with the two transposon systems described above. The transpososome method mentioned above can also be used to make random mutations in mycobacterial genomes (71), but a tagging system has not been developed for this method either, limiting its usefulness in screening for mutants with attenuated virulence.

(iii) Complementation. Genetic complementation has also been used to identify *M. tuberculosis* virulence genes. These studies use *M. tuberculosis* strains that are known to be avirulent or nonpathogenic as recipients for genes that can be selected on the basis of encoding a virulence phenotype, using assays described earlier in this review. In vivo complementation has been made possible by using integration-proficient vectors, which allow the stable propagation of genomic libraries as well as individual genes in bacteria during animal infections (160). It was shown that the random cloning of an *M. tuberculosis* cosmid library into an avirulent *M. bovis* strain localized the attenuating mutation in *sigA*, encoding the major mycobacterial sigma factor, since the wild-type *sigA* restored virulence in a guinea pig morbidity (spleen focus) assay (56). A similar *M. tuberculosis* cosmid library transformed into the avirulent *M. tuberculosis* strain H37Ra permitted the isolation of a DNA fragment that increased bacterial survival in mouse spleens but not lungs (212). The nonpathogen *M. smegmatis* was also used as the host in similar experiments. In one case, a cosmid library made with *M. bovis* BCG DNA was transformed into *M. smegmatis*, allowing the identification of a chromosomal fragment that modestly increased bacterial survival in mouse spleens and in mouse peritoneal macrophages (87). An *M. tuberculosis* plasmid library was used to identify *eis*, a gene that increased the survival of *M. smegmatis* in the human macrophage-like cell line U937 (310), and another plasmid library allowed the isolation of *mce1*, a gene that increases the entry of *E. coli* into HeLa cells that are nonphagocytic (7). A related approach but using a specific gene has also been tested. The *M. leprae* thioredoxin-thioredoxin reductase gene, cloned into a plasmid vector, was able to increase the survival of *M. smegmatis* in human MDMs (313).

(iv) Antisense methods. Antisense RNAs are used to reduce the expression of specific genes because they prevent the translation of the mRNAs to which they are complementary. They are especially useful in systems where gene inactivation is difficult and also when genes are essential because antisense inhibition of translation is rarely, if ever, complete. A general system for conditionally controlling the production of antisense RNA in mycobacteria was developed, using the regulatable acetamide/acetamidase system (210). In a demonstration of the usefulness of this method, a prototrophic *M. smegmatis* stain was made into a histidine auxotroph when a *hisD5* antisense RNA was induced by acetamide. Other applications of the antisense method were in *M. bovis* to lower the levels of AhpC (315) and in *M. tuberculosis* H37Rv to reduce the amounts of SodA (82). These are discussed later in this review. A related antisense approach, but using phosphorothioate antisense deoxyoligonucleotides, was used to decrease levels of the *M. tuberculosis* glutamine synthetase in growing cells (121).

Other (nongenetic) methods. Gene inactivation, either directed or global, and the subsequent analysis of mutant phenotypes is the most straightforward way to identify and characterize genes and proteins that are involved in a specific process and, for *M. tuberculosis*, is virulence. A major problem with this approach is that some genes may be essential and cannot be disrupted. Thus, methods that do not rely on the absence of a function are also useful, and these "nongenetic" screens usually rely on the differential expression of genes and their products in different environments. As in the genetic methods, they can be used to characterize the expression of individual genes identified by other means or can function in global searches for genes that show the desired pattern of gene expression. The output of these methods can be enzyme activity using reporter genes, levels of RNA or proteins, and in some cases direct selection of genes using a selectable or screenable phenotype.

(i) Reporter fusions and promoter traps. A general method for studying bacterial gene expression is that of reporter gene methodology. This utilizes plasmid vectors that contain a promoterless gene encoding a protein that, in most cases, catalyzes an easily assayable enzymatic reaction. Specific promoter sequences from known genes or random chromosomal fragments are cloned upstream of the promoterless reporter gene, and this construct is electroporated into the mycobacterial cell so that the activity of the gene in question can be simply measured under different in vitro conditions and for *M. tuberculosis* during infections. Among the reporter genes used for this purpose in mycobacteria and the proteins they encode are *lacZ* (β-galactosidase), *xylE* (catechol-2,3-dioxygenase), *phoA* (alkaline phosphatase), *lux* (luciferase), *gfp* (green fluorescence protein [GFP]), and *cat* (chloramphenicol acetyltransferase), and plasmid vectors with these reporters have been described previously (reviewed in reference 294). In addition to measuring the

levels of individual *M. tuberculosis* genes under different conditions, e.g., during infection of macrophages (66, 128) and iron starvation (243), reporter technology has permitted the selection or screening of mycobacterial genes that are differentially expressed, a technique known as promoter trap cloning. The *cat* gene was used for the cloning of physiologically functional promoters of *M. smegmatis* and *M. tuberculosis* by means of chloramphenicol resistance (64), and *lacZ* screening on solid indicator media allowed the cloning of *M. tuberculosis* H37Rv genes that were induced by NO (128). *M. marinum* genes that are more highly expressed during infection of macrophages were also cloned by differential fluorescence of *gfp* fusions, using fluorescence-activated cell sorting (16, 231), and a similar method was used for *M. bovis* BCG (288).

A novel promoter trap system has been recently developed that has allowed the cloning of several *M. tuberculosis* genes induced in human macrophages (75). This selection system is similar in principle to in vitro expression technology, which was initially developed to identify virulence genes in gram-negative pathogens (171, 172). The *M. tuberculosis* system is based on the observation that overexpression of the *inhA* gene in mycobacteria confers resistance to the front-line antitubercular drug INH (11). This occurs because the mode of action of INH, when activated, is to irreversibly bind to and inactivate the essential InhA protein that is involved in mycolic acid biosynthesisis (230). Thus, the drug will be sequestered as it is bound by the high levels of InhA, resulting in phenotypic INH resistance. The promoter trap selection uses a plasmid in which a library of *M. tuberculosis* DNA fragments can be inserted upstream of a promoterless *inhA* gene. *M. tuberculosis* cells containing plasmids with functional promoters are then selected by growth in the presence of INH under different conditions, e.g., during macrophage infections (75) and in the lungs of *M. tuberculosis*-infected mouse lungs (E. Dubnau and I. Smith, unpublished results).

(ii) Hybridization-based methods. Several methods that characterize *M. tuberculosis* mRNAs have been described, and these can be used to quantitate the expression of specific genes or to globally identify and measure many RNA transcripts. Generally these techniques are based on the production of cDNA from RNA, using specific primers for known genes or random priming for unknown ones, frequently followed by PCR amplification. Among these methods used with *M. tuberculosis* to measure individual transcripts are classical reverse transcriptase followed by PCR amplification (RT-PCR), which was used to examine differences in levels of specific mRNAs in *M. bovis* BCG after heat shock (213) and during human macrophage (43) and animal infections (136). A similar study was carried out with several bacterial genes during *M. tuberculosis* H37Rv infection of human macrophages (184).

One of the problems in the standard RT-PCR method when used to measure specific *M. tuberculosis* RNA transcripts during infection is that the products of the PCR amplification step are frequently not unique because of contaminating host RNA. Quantitation of the transcripts is also not always reliable. A variation of RT-PCR has been developed that uses fluorescent molecular beacons to measure the levels of the PCR product in real time with a spectrophotofluorometric thermal cycler (180). Molecular beacons are single-stranded DNA probes that fluoresce only when hybridized to a single-stranded DNA sequence (296). The hairpin structure of the molecular beacon gives extremely high specificity to its annealing with its target, so that a 1-base mismatch will eliminate this interaction (295, 296). This highly specific and accurate assay has been used to measure levels of several sigma factor gene mRNAs when *M. tuberculosis* H37Rv is grown under different stress conditions (179) and to quantitate the mRNAs of various *M. tuberculosis* genes during infections of human macrophages (75) and mouse lungs (264).

Amplification-based hybridization techniques have also been used to globally identify *M. tuberculosis* transcripts. Examples of these procedures are (i) differential display, which has allowed the identification of several genes that are differentially expressed in *M. tuberculosis* H37Rv grown in broth and in its avirulent descendant, *M. tuberculosis* H37Ra (242); (ii) a cDNA method (DECAL), which eliminates abundant RNAs which could interfere with the specificity of PCR amplification and also optimizes various parameters and was used to identify transcripts induced after antibiotic treatment of *M. tuberculosis* growing in broth culture (2); (iii) a method involving random cDNA synthesis followed by subtractive hybridization and PCR amplification (SCOTS), which was used to identify *M. tuberculosis* (115) and *M. avium* (135) genes expressed in human macrophages.

An important benefit of the completion of the *M. tuberculosis* genome and its annotation has been the development of DNA arrays that allow expression profiling of all the *M. tuberculosis* genes. DNA arrays (DNA chips) are dense grids of DNA bound to a solid matrix that can be probed with a complex mixture of labeled cDNAs. The major advantages of microarrays over older technology are the increase in the number of genes being analyzed, the substantial reduction in sample size requirements, and the use of fluorescence detection schemes that provide high-signal-to-noise ouputs. Microarrays can be used for genotyping, as well as for expression profiling studies.

Two major DNA chip platforms are currently in use, and many more are in development. The most widely used method is based on a technique (spotting arrays) invented by Pat Brown and colleagues at Stanford University (84). It begins with the isolation and placement of individual (500- to 5,000-bp) PCR products (representing individual genes) on a small glass microscope slide in an array format with each gene occupying a unique location. The PCR products are robotically printed and bonded onto the glass slide, denatured, and hybridized to two fluorescence-labeled samples representing the expressed mRNAs from two different cell types or conditions. Generally the fluorescent dyes Cy5 and Cy3 are used to label cDNA made from the input RNA. The reference sample is prepared by isolating mRNA from cells growing under one condition and generating a fluorescently labeled probe. A second sample of mRNA is extracted from differently treated cells and used to generate a second probe labeled with the other colored fluorescent molecule. Labeled cDNAs from the two cell samples are simultaneously applied to a single microarray, where they competitively react with the arrayed cDNA molecules. Each position on the microarray can then be scanned for the two fluorescent colors, using fluorescence detection coupled to microscanning instrumentation. Since the fluorescence intensity is proportional to the expression level of a gene in a

particular sample, determining the ratio of the two fluorescent intensities provides a highly accurate and quantitative measurement of differences in the relative levels of gene expression in the two samples. The Brown-type platform has been used for many prokaryotic systems including pathogens, and an ORF array of essentially all of the *M. tuberculosis* genes was first made by Gray Schoolnik's group at Stanford University (256). This and more recently developed arrays have been used for several different studies, including a genotyping analysis of the evolutionary relationship between *M. bovis* BCG vaccine strains (20) and a study to localize insertion sites for the IS *6110* element (154). TB DNA arrays have been used for a global gene expression analysis of *M. tuberculosis* that has been exposed to the antitubercular drug INH (314) and to acidic growth conditions (93) and heat shock (276), as well for a study of the effects of mutations in *M. tuberculosis* genes encoding transcriptional regulatory proteins on global gene expression. Among the mutants analyzed are some with disrupted genes encoding sigma factors (151, 181, 182), two-component signaling proteins (263, 312; S. Walters and I. Smith, unpublished results), and the major *M. tuberculosis* iron flux regulator, IdeR (244).

A second microarray platform, which essentially differs in the manufacturing of the microarray, is available exclusively from Affymetrix Inc. Instead of using PCR products, approximately 15 pairs of overlapping oligonucleotide 25-mers are synthesized for every gene. For each pair, one represents the wild-type sequence and the second contains a single-nucleotide mismatch located in the center of the oligonucleotide sequence. Each oligonucleotide is coupled on a unique glass wafer as it is synthesized in parallel using photolithographic methods. An Affymetryx chip for for the complete *M. tuberculosis* genome has not been described yet, but one has been developed for genotyping that has DNA sequences corresponding to several alleles of the *M. tuberculosis rpoB* gene and multiple sequences of the 16S rRNA gene of the *M. tuberculosis rrnA* cistron (289). In addition, two recent studies used Affymetrix chips made with mouse (83) and human (198) genes to study mouse and human macrophage responses to *M. tuberculosis* infection. This significant work is discussed later in this article.

(iii) Proteomics. Proteomic analyses have also provided global information on gene expression by measuring the levels of proteins during different *M. tuberculosis* growth conditions such as iron starvation (44) and macrophage infections (159). Two-dimensional gene electrophoresis technology to resolve many of the *M. tuberculosis* proteins, together with mass spectrophotometric determinations that can identify these molecules (147, 194), has also been used to study proteins whose presence is not predicted by the DNA sequence (146), some of whose levels are regulated by iron starvation (318) and low levels of oxygen (246), and to identify proteins that are released into the extracellular media when *M. tuberculosis* is grown in broth culture (271). Determination of individual protein levels on a global scale is a very important complementary procedure to DNA array analyses because levels of proteins can frequently be determined by regulation at posttranscriptional steps, e.g., intein excision of polypeptides from *M. tuberculosis* RecA precursor, as discussed above.

However, knowledge of the levels of specific mRNAs and the proteins they encode, obtained from DNA arrays and proteomic determinations, respectively, still does not give a complete picture of the levels of functional proteins when *M. tuberculosis* or any cell is exposed to different conditions. The activities of many proteins can also be controlled by various posttranslational processes which themselves may be regulated. Among these are protein-protein interactions between certain sigma factors, anti-sigma factors, and anti-anti-sigma factors (reviewed in reference 123) which also occur in *M. tuberculosis* (19; S. Rodrigue et al., unpublished experiments). In addition, the activity of proteins can be controlled by covalent modification, e.g., the phosphorylation of two-component response regulators (129) and the activation of some DNA binding proteins by the electrostatic binding of divalent metals, the best-known examples being the activation of proteins in the Fur (103) and DtxR (285) families by iron and related metals. IdeR, a mycobacterial protein in the DtxR family, regulates genes involved in iron acquisition and storage (110, 244) and requires the binding of divalent metal ions like iron for its interaction with specific DNA sequences (255).

Validation of results obtained from genetic and gene expression studies. The use of the techniques described in this section for studying the genetics of *M. tuberculosis* have already identified many genes that are important for virulence or some physiological property or are differentially expressed under various conditions, including infection of macrophages or animal models. In many of these studies, the initial results must be considered preliminary until they are validated by separate experiments.

In the case of the genetic experiments in which a mutated phenotype is obtained when a gene is inactivated, it is essential to secondarily construct a mutant strain that contains a wild-type copy of the gene, ideally integrated into the chromosome in single copy at an ectopic site and under the control of its natural promoter. For this purpose, plasmid vectors that utilize the *att* system of mycobacteriophage L5 for ectopic integration of DNA are widely used (160). The complemented mutant strain should show reversion of all mutant phenotypes to those of the wild-type parent. Only in this way can one be sure that the phenotypes observed after a gene is inactivated are specifically due to the mutation. Since many genes are in polycistronic clusters, it is always possible that the phenotype associated with a mutation is due to a polar effect on a downstream gene. Even if a gene is monocistronic and polarity effects can be ruled out, other events not directly due to the mutation can occur when a gene is disrupted. An example of this occurred during the construction of an *M. tuberculosis* mutant with a disrupted *ideR* gene (244). The mutant had very clear in vitro phenotypes, such as deregulated (iron-independent) siderophore production and a weakened response to oxidative stress, that were completely restored to those of the wild type in a complemented mutant strain. The poor growth of the mutant in human macrophages was also returned to wild-type levels when the wild-type *ideR* gene was present. However, the extremely attenuated growth phenotype of the mutant in mice, manifested by a bacterial load that was 4 log units lower than that of the wild type, was not restored in the complemented strain (G. M. Rodriguez and I. Smith, unpublished experiments). Other experiments then showed that the mutant and

the complemented mutant strains were deficient in the uptake of iron so that they could not grow in low-iron-containing media. Evidently, a suppressor mutation that affected the ability of *M. tuberculosis* to acquire iron had occurred during the disruption of *ideR*, which is now thought to be an essential gene. Thus, the initial conclusion that *ideR* was necessary for *M. tuberculosis* growth in mice was wrong and only by using the complemented mutant could the erroneous conclusion be corrected (244). The majority of studies in which *M. tuberculosis* genes are inactivated do show that complementation with the wild-type gene restores all phenotypes to the normal situation, but there some examples in the literature in which this was not done. Since many of the *M. tuberculosis* gene inactivation studies concern virulence phenotypes, any conclusion that a gene is important for virulence should be interpreted with caution unless this phenotype can be complemented with the wild-type gene.

Results obtained from gene expression studies, especially those done on a global scale, should be considered preliminary until they are validated using a different technique, in which the levels of individual mRNAs are directly measured by RT-PCR, Northern, or primer extension analysis. In DNA array analyses, since the levels of 4,000 genes are being compared usually as a ratio between two conditions or bacterial strains, many factors such as background noise (84) and statistical considerations (137, 161) may give erroneous results for the values obtained with specific genes. Validation for representative genes is routinely done in most *M. tuberculosis* DNA array studies (181, 182, 314).

Similarly, results obtained in experiments in which gene expression is studied with multicopy plasmids should be confirmed. The use of multicopy plasmids for reporter fusions (66) or promoter traps (16, 75, 231) simplifies cloning, but frequently chromosomal genes are regulated differently when contained on plasmids, presumably because the DNA conformation is altered. An example of this is the *M. tuberculosis hsp60* gene, which is induced after heat shock but is constitutive when present on a multicopy plasmid (280). In work previously discussed, a promoter trap carried on a multicopy plasmid during *M. tuberculosis* infection of human macrophages was used to identify 43 genes as being induced during the infection based on their differential resistance to INH (75). To validate these results, molecular beacon RT-PCR was used to measure the mRNA of a subset of these genes, and it was found that 8 of the 13 tested genes were induced during infection of macrophages by normal (plasmid-free) *M. tuberculosis* H37Rv (75). This result indicates that the InhA promoter trap was useful in identifying some genes that have the desired gene expression pattern, allowing further investigation of their roles in virulence, but that false-positives are also selected.

M. TUBERCULOSIS VIRULENCE FACTORS

As described above, the virulence of *M. tuberculosis* can be measured during the infection of macrophages and animals, using several different assays, and various strategies have been developed to make mutations in *M. tuberculosis* genes. A combination of these methods has allowed researchers to identify several genes that are important for various aspects of *M. tuberculosis* pathogenicity, and this part of the review discusses some of these genes and the cellular components they encode. They are grouped below according to the known or predicted function of the proteins, based on DNA sequence annotation. Some genes have also been identified as being upregulated during infection. In most cases, their essentiality for virulence has not been established by gene inactivation studies, and some of them are briefly discussed in the context of related genes that are known to be essential to this process.

Cell Secretion and Envelope Function

In this category are listed genes encoding proteins that are expected to be exposed to the environment in which *M. tuberculosis* grows, either in culture media or in the mycophagosome. Among these are secreted proteins and enzymes that play a role in the synthesis of various cell surface molecules. The structure of the mycobacterial cell wall and envelope is extremely complex, and a discussion of this important and unique barrier that separates *M. tuberculosis* from its external enviroment is beyond the scope of this review. However, there are some excellent reviews on this subject (36, 61).

Culture filtrate proteins. *M. tuberculosis* culture filtrate proteins (CFPs) are those found in the culture medium in which *M. tuberculosis* is grown. They are so defined because mechanisms of secretion are not known for all these proteins, of which there are approximately 200 (271). In addition, some of these proteins are associated with cells, so the definition of CFP is an operational one. CFPs are actively studied by many *M. tuberculosis* researchers, since many are recognized by the sera of TB patients. It has also been postulated that live attenuated *M. tuberculosis* vaccines are better than those made from heat-killed cells because during growth in the host, *M. tuberculosis* releases CFPs that stimulate host immune mechanisms (3). Interestingly, KatG (catalase-peroxidase) and SodA (superoxide dismutase), enzymes that degrade ROIs and are important for *M. tuberculosis* survival during infection (as discussed below), are found in the culture filtrate. The significance of this localization is not known, but it has been speculated that the location may allow more efficient detoxification of harmful molecules produced by the host in the phagosome (35). Many of the proteins founds in the culture filtrate, e.g., SodA KatG, and GlnA (glutamine synthase), do not have leader sequences that are usually involved in protein secretion, but the fact that they were be released from cells early in growth suggested that this localization was physiological and not dependent on cell lysis. However, more recent experiments show that only proteins highly expressed in *M. tuberculosis*, such as GlnA and SodA, which are also very stable, are found in early culture filtrates, while less abundant intracellular proteins or those that are unstable are not found extracellularly (293). These results strongly suggest that the presence of many proteins in culture filtrates, especially those with missing leader sequences, is caused by bacterial leakage or lysis.

(i) HspX (Rv2031c, *hspX*). HspX, also known as Acr, i.e., the α-crystalline protein homolog or the 16-kDa protein, is a major *M. tuberculosis* antigen recognized by the sera of a high proportion of TB patients and is induced under anoxic conditions (306). This gene is also induced in human THP-1 macrophages, and a *M. tuberculosis* mutant in which the *hspX* gene

was inactivated was severely attenuated for growth in these macrophages (324). It is postulated that the chaperone-like HspX is an important controlling element in *M. tuberculosis* latency or persistence, since overexpression of the protein inhibits *M. tuberculosis* growth (325). As discussed below, the induction of *hspX* under anoxic conditions requires the response regulator Rv3133c (263).

(ii) Esat6/CF-10 (Rv3875, Rv3874). The Esat6 and CF-10 proteins are members of the Esat6 family of related small secreted proteins found in *M. tuberculosis* culture filtrates. Both proteins are immunodominant antigens that are recognized by the sera in a majority of TB patients (265). A mutation that disrupts both closely linked genes in *M. bovis* results in severe attenuation in a guinea pig model of infection, using the criteria of histopathology in several tissues and bacterial load in spleens (305). It is not known which of the two genes (or both) are necessary for the virulence of *M. bovis* in this animal model. Rv3874 and Rv3875 are located in the RD1 deletion region, the first deletion found when comparing the genome of a wild-type *M. bovis* strain and *M. bovis* BCG (170). RD1 contains the structural genes for nine proteins, Rv3971 through Rv23979 (53). This region is found in all virulent *M. tuberculosis* and *M. bovis* strains but is the only deletion found in all *M. bovis* BCG strains (38), initially suggesting that some of the genes in this region were important for virulence. This has now been confirmed by two independent series of experiments. In one case, using a knockout strategy, a deletion of the RDI region was made in *M. tuberculosis* H37Rv, and the resulting mutant had the same attenuated virulence phenotype as the classical *M. bovis* BCG strains (162). The other experiments used a knockin approach, in which the RD-1 region was inserted into the chromosome of *M. bovis* BCG. These studies showed that the complemented strain was much more virulent than its attenuated parent (227). It is not yet known whether any of the other genes of the RD1 region are necessary for virulence. Significantly, the avirulent *M. microti*, which has been used as a live vaccine strain, also contains the RD1 deletion, and its virulence is increased when the RD1 region from *M. tuberculosis* is introduced by knockin methods (227).

The Esat6 and CF-10 genes are cotranscribed in *M. tuberculosis* (27); when coexpressed in *E. coli*, they form a tight 1:1 complex (239). The members of the *M. tuberculosis* Esat6 family are frequently found in the same type of genomic arrangement since many of their genes are found in closely linked pairs in the *M. tuberculosis* genome (53). This suggests that similar 1:1 complexes will also be formed. Another member of the Esat6 family, Rv0288, has been identified as being differentially expressed in mouse lungs by using a promoter trap screen, and it is also induced in macrophages, as determined by RT-PCR analysis (Dubnau and Smith, unpublished). TB10.4, the product of Rv0288, is recognized by sera from 70% of TB patients, and T cells from this cohort show a strong cytokine response (IFN-γ release) when the protein is presented (265). The role of the virulence of Rv0288 in virulence has not been analyzed yet, but experiments are in progress. Rv0288 is also found adjacent to another gene, Rv0287, that encodes a member of the Esat6 family, suggesting a complex of these two proteins.

(iii) 19-kD protein (Rv3763, *lpqH*). The 19-kDa protein is an immunodominant antigen that is recognized by T cells and sera from TB patients. When *M. tuberculosis* enters macrophages and other phagocytic cells, this surface-exposed glycolipoprotein is thought to cause host signaling events as it interacts with TLR2 (201, 287). However, there are confusing and contradictory reports regarding host responses to this protein. It was originally noted that *M. tuberculosis* strain I2646 did not produce the 19-kDa protein, and subsequent DNA sequence analyses demonstrated that the I2646 *lpqH* gene was disrupted. The strain was rapidly cleared from the lungs and spleen of infected mice, a SGIV phenotype. Adding back the wild-type *M. tuberculosis* gene to *M. tuberculosis* I2646 allowed growth in lungs that was similar to the growth of standard wild-type strains, strongly suggesting that the 19-kDa protein is essential for virulence (158). Later experiments were less conclusive because was shown that mutations in the gene encoding the 19-kDa protein had no effect on *M. intracellulare* virulence (173) and that the addition of genes encoding the 19-kDa protein to nonpathogenic mycobacteria actually lowered their efficacy as vaccines (320). In addition, it was found that neither overexpressing the 19-kDa protein nor deleting its structural gene in *M. bovis* BCG had any effects on the ability of these strains to serve as protective vaccines in a mouse infection model (321). Equally uncertain is the effect of the protein on host responses during *M. tuberculosis* infection of macrophages. It has been reported that addition of the purified 19-kDa protein to human MDMs causes upregulation of the important Th1 cytokine IL-12 (37). Similarly, addition of the 19-kDa protein can activate human neutrophils (199). On the other hand, when the gene for the 19-kDa protein was introduced into *M. smegmatis* and the recombinant strain was used to infect human MDMs, IL-12 production was inhibited (224). When *M. tuberculosis* is used to infect murine macrophages, IL-12 levels are not elevated (126, 198), suggesting that the context in which the 19-kDa protein is presented is a key factor in macrophage response. Interestingly, murine dendritic cells infected with *M. tuberculosis* do show elevated levels of IL-12 (126). Another report demonstrated that the addition of the 19-kDa protein to murine macrophages causes the inhibition of MHC-II expression and MHC-II antigen processing (201), also suggesting that macrophage responses to *M. tuberculosis* infection are downregulated.

(iv) Glutamine synthase (Rv2220, *glnA1*). The inclusion of glutamine synthase is an operational one, since (as discussed above) its presence in culture filtrates probably results from cell leakage and lysis. *glnA1* mutations have not been made in *M. tuberculosis*, but the specific glutamine synthase inhibitor L-methionine-*SR*-sulfoximine (MSO) inhibits the growth of *M. tuberculosis* in vitro and in human MDMs (120) as well as in guinea pigs (119) but has no effect on nonpathogenic mycobacteria or nonbacterial microorganisms. In addition to its essential role in nitrogen metabolism, MSO inhibition studies have shown that the *M. tuberculosis* glutamine synthase is involved in the synthesis of a poly-L-glutamate-glutamine cell wall component found in pathogenic mycobacteria (120). These results suggest that this enzyme is a good target for the development of new drugs with less toxicity than MSO for the mammalian host.

Cell surface components. As discussed above, the mycobacterial cell wall and envelope is a complex structure containing many proteins, lipids, and carbohydrates, many of which are found only in these bacteria. Is a subset of these components

are unique to pathogenic mycobacteria and thus are expected to be excellent targets for further investigations of *M. tuberculosis* virulence.

(i) Erp (Rv3810, *erp*). Erp is a surface-located protein that was originally identified by means of a *phoA* fusion strategy to identify secreted *M. tuberculosis* proteins (25, 26). The protein is similar to an exported 28-kDa antigen (the PLGTS antigen) in *M. leprae* and is not found in nonpathogenic mycobacteria. The *M. tuberculosis* protein has six tandem repeats with the sequence (PA/G)LTS, which is similar to the *M. leprae* protein. The *M. tuberculosis erp* gene was inactivated by a two-step plasmid procedure, and the mutant showed attenuation of growth in primary murine macrophages and was also attenuated in the lungs and spleen of infected mice, exhibiting a SGIV phenotype. A similar *erp* mutation was made in *M. bovis* BCG, and this mutant showed a PER attenuation phenotype in mice (26). The function of the protein is unknown because the mutant shows normal growth in vitro but mutant bacteria recovered from infected mice grow much more slowly that the wild-type and complemented mutant strains.

(ii) Mas (Rv2940c, *mas*). *mas* encodes mycocerosic aid synthase, an enzyme that catalyzes the synthesis of long-chain, multiply methylated branched fatty acids, called mycocerosic acids, that are found only in pathogenic mycobacteria. The *mas* gene was disrupted in *M. bovis* BCG, using a linear DNA construct, and was shown to be deficient in the synthesis of mycocerosic acids and their phthiocerol dimycoserate (PDIM) derivatives (8). No data on the virulence phenotypes of the *mas* mutant were presented, but citation of unpublished experiments mentioned that it was attenuated for growth in macrophages and mice. This is consistent with the results of other published studies that are discussed in the following sections. The Kolattukudy group has also created mutations in genes encoding related cell wall components, but their virulence phenotypes have not been reported (8, 73).

(iii) FadD26 (Rv2930, *fadD26*). FadD26 was originally annotated as an acyl coenzyme A (acyl-CoA) synthetase involved in fatty acid degradation. It was inactivated by the STM procedure in *M. tuberculosis* strain 103, and when the mutant was individually tested for bacterial load at one time point, 3 weeks after infection, it grew approximately 2 log units less well in mouse lungs than did the wild-type strain. In the same studies, *M. bovis* BCG grew 3 log units less well than did the wild type (46). Similar mouse results were obtained by another group using STM technology with *M. tuberculosis* H37Rv; the *fadD26* mutant in this study exhibited a GIV phenotype (59). In this latter case, the transposon inserted in the promoter region of *fadD26* and the disruption also affected the expression of the downstream *ppsA* to *ppsE* operon (Rv2931 to 2935) encoding a polyketide synthase required for phthiocerol biosythesis (8), and one of the mutant phenotypes is the absence of PMID. It is not known whether the phenotypes of the *fadD26* transposon insertion mutations are solely due to a polar effect on the downstream *pps* operon or also reflect a role for FadD26 in these processes. However, the close linkage between *fad26* and genes for PMID synthesis, including *mas*, suggests that FadD26 may have a synthetic rather than a degradative function, as originally annotated.

(iv) FadD28 (Rv2941, *fadD28*). *fadD28* was found in one of the STM searches that identified *fadD26* (59). Like the latter gene, it was also annotated as a fatty acid CoA synthase, and the *fadD28* mutant has a similar GIV phenotype in mice. *fadD28* is in the *mas* region, and the mutant also does not make PDIM.

(v) MmpL7 (Rv2942, *mmpL7*). MmpL7 was identified in STM transposon searches as being important for *M. tuberculosis* virulence (46, 59). This protein is a member of a large group of related proteins, and one of the phenotypes of the mutant is the failure to transport PMID, although it does synthesize this complex molecule (45, 59). The mutant is attenuated for growth in mice exhibiting a GIV phenotype. Other members of the *mmpL* family were identified in one of the transposon searches, as were several other genes but they are not discussed here because they not yet been studied in great detail (46).

(vi) FbpA (Rv3804c, *fbpA*). Mycobacteria have three mycolyl-transferase enzymes, encoded by three genes, *fbpA*, *fbpB*, and *fbpC*, that transfer long-chain mycolic acids to trehalose derivatives, and the proteins can also bind the cell matrix protein fibronectin (22). The Fbp proteins are also found in the culture filtrate and are also known as the antigen 85A, 85B, and 85C complex or the 30- to 32-kDa proteins. The three *fbp* genes have been separately inactivated, but only the *M. tuberculosis fbpA* mutant, made with a linear DNA construct, showed severely attenuated growth phenotypes in human and murine macrophages (5). The observation that these proteins are immunodominant has led to the creation of a new live vaccine that was made by introducing the *M. tuberculosis fbpB* gene into *M. bovis* BCG (133). This recombinant strain shows better protection against virulent *M. tuberculosis* infection than does the parent BCG strain in a guinea pig model. This vaccine strategy has not been used with the *M. tuberculosis fbpA* gene, but presumably this will be done, given the reported virulence phenotype of the *fbpA* mutant.

(vii) MmaA4 (Rv0642c, *mmaA4*). A gene cluster in *M. tuberculosis* has been described that encodes four closely related methyltransferases whose function is to form methoxy and keto derivatives of the meromycolic acid chain that are unique to members of the *M. tuberculosis* complex (76, 323). It was postulated that the reaction catalyzed by the methyltransferase encoded by *mmaA4*, i.e., a methylation of a double bond, is the initial step for all subsequent derivatizations of meromycolic acids (76). To verify this and to see the effects of this mutation on *M. tuberculosis* virulence, the *M. tuberculosis mmaA4* gene was inactivated by a two-step plasmid procedure (74). The mutant grows normally but does not make the methoxy- and ketomycolates, as originally predicted. In addition, it shows marked cell wall alteration since it is less permeable to various compounds such as glycerol and chenodeoxycholate but is more resistant to oxidative stress than the wild-type *M. tuberculosis* H37Rv. The mutant strain was attenuated in mice showing a GIV phenotype (74).

(viii) PcaA (Rv0470c, *pcaA*). PcaA is a methyltransferase that forms cyclopropane residues in mycolic acids. It was originally detected on the basis of the unusual colony morphology of an *M. bovis* BCG transposon mutant (108). Microscopic examination of the mutant showed a corded structure of the clumped bacterial cells, and sequence analyses of the mutated gene showed it was a member of a family of proteins that introduce cyclopropane residues into mycolic acids. To analyze

the role of PcaA in *M. tuberculosis* physiology and virulence, the structural gene was inactivated by a bacteriophage-mediated system (108). The colonial and microscopic phenotypes of the mutant were similar to those of the *M. bovis* BCG *pcaA* mutant, and biochemical studies indicated that the role of the enzyme was to synthesize the proximal cyclopropane ring of the α-mycolate chain of mycolic acids. While the BCG mutant was cleared more rapidly from the lungs than its parent, the growth of the *M. tuberculosis* mutant was essentially similar to that of the wild type. However, the *M. tuberculosis pcaA* mutant was less virulent in a mortality assay, in which five of five mice infected with the wild type all died after 219 days while all five mice infected with the mutant were still alive at this time. Microscopic observations showed much less pathology in the lungs of mice infected with the mutant strain than in the lungs of those infected with the wild-type strain.

(ix) OmpA (Rv0899, *ompA*). A porin-like protien, OmpA, has been found in *M. tuberculosis* H37Rv, and it can form pores in liposomes, a property of porin family proteins (261). *ompA* expression was induced by low pH and also during growth in macrophages, and an *ompA* mutant, made by a two-step plasmid procedure, demonstrated the following phenotypes: it showed delayed growth at acid pH but ultimately grew at wild-type levels; it could not take up small molecules like serine at low pH; it was attenuated for growth in both human and murine macrophages; and in mice it showed a GIV phenotype in both lungs and spleens (237). This is an extremely interesting result, suggesting that the environment faced by *M. tuberculosis* during infection is acidic and that OmpA plays a role in the bacterial reponse to this condition. One caveat to this hypothesis is that true complementation experiments were not performed in these studies, i.e., introducing the wild-type *ompA* gene into the mutant to see if virulence was restored to normal levels. As discussed elsewhere in this review, complementation is essential for concluding that a particular phenotype is caused by a mutation.

(x) HbhA (Rv0475, *hbhA*). HbhA is a heparin-binding hemagglutin protein (220) that is localized on the surface of virulent mycobacteria but is not found in *M. smegmatis. hbhA* was inactivated in *M. tuberculosis* 103 by the plasmid *ts* replicon-sucrose two-step selection method, and the virulence phenotypes of the mutant were tested (220). The mutant exhibited wild-type ability to be phagocytosed by and to grow in murine or human macrophages. However, it was taken up poorly by pneumocytic cells, although the bacterial generation time was normal intracellularly. The mutant grew normally in the lungs of infected mice but had a longer generation time and reached a lower bacterial load in spleens compared to the wild-type and complemented mutant strains. The unique properties of this mutant indicate that HbhA is important for *M. tuberculosis* interaction with pneumocytes and also that this interaction may play a role in extrapulmonary dissemination.

(xi) LAM. LAM is included in this list of virulence factors because of its importance as an immunomodulator as detemined by experiments similar to those described above for the 19-kDa protein. LAM, a complex glycolipid that contains repeating arabinose-mannose disaccharide subunits, is a major component of the *M. tuberculosis* cell wall (138). Addition of LAM to murine macrophages depresses IFN-γ production, which, in turn, blocks the expression of IFN-γ-induced genes (49). LAM can also scavenge oxygen radicals, in vitro, and inhibits the host protein kinase C. These multiple phenotypes suggest that LAM functions to downmodulate host responses to *M. tuberculosis* infection, protecting the bacterium from potentially lethal mechanisms like the respiratory burst (49). *M. tuberculosis* mutants that do not make components of LAM and that prevent its synthesis have not been isolated yet, but these should be valuable for the insights they will provide into *M. tuberculosis*-host interactions.

Enzymes Involved in General Cellular Metabolism

Since many pathogens become starved for certain essential nutrients and cofactors, e.g., carbon sources, amino acids, purines, pyrimidines, and divalent metals like Mg^{2+} and Fe^{2+}, during infection, *M. tuberculosis* researchers have systematically created mutations in genes encoding enzymes in the biosynthetic/degradative pathways and acquisition systems for some of these factors. In addition, mutations have been created in genes encoding respiratory enzymes and enzymes that protect against oxidative stresses occurring during normal aerobic respiration, as well those created by infected hosts.

Lipid and fatty acid metabolism. As discussed above, observations made approximately 50 years ago indicated that *M. tuberculosis* shifts from a metabolism that preferentially uses carbohydrates when growing in vitro to one that utilizes fatty acids when growing in the infected host (258, 259). These old observations are supported by more recent work, e.g., the complete sequencing and annotation of the *M. tuberculosis* genome, in which over 200 genes were annotated as being involved in fatty acid metabolism (53), and in work discussed directly below.

(i) Icl (Rv0467, *icl* or *aceA*). Icl (isocitrate lyase) is an enzyme that converts isocitrate to succinate in the glyoxalate shunt. This allows bacteria and plants to grow on acetate or fatty acids as sole carbon sources since the glyoxalate shunt provides a source of carbon that can enter the Krebs cycle. Initial observations made using an in vitro model to study *M. tuberculosis* persistence showed that isocitrate lyase activity increases dramatically as the cells reach stationary phase (307) and that its mRNA level increases when *M. tuberculosis* infects human macrophages (75, 115). The *M. tuberculosis* Erdman *icl* was inactivated by means of allelic replacement using a two-step plasmid system, and the mutant cannot grow on C_2 carbon sources (188). It initially grows normally in mice but stops growing in lungs and is cleared at the time when cell-mediated immunity is initiated, a PER phenotype (188). The mutant has wild-type growth in IFN-γ knockout mice and in inactivated primary murine macrophages but is killed more rapidly than the wild type when these macrophages are activated with IFN-γ and lipopolysaccharide. Additional evidence indicating the importance of isocitrate lyase includes the observation that *icl* mRNA levels increase in the lungs of *M. tuberculosis*-infected mice as the infection progresses (264).

(ii) LipF (Rv3487c, *lipF*). LipF is annotated as a lipase/esterase that may function in lipid degradation. It was identified in one of the STM experiments that found *fadD26* (discussed above) and had a similar attenuated phenotype in mice (46). Nothing more is known about the function of this protein or why it is essential for bacterial survival in vivo.

(iii) FadD33 (Rv1345, *fadD33*). There are 36 genes annotated in the *M. tuberculosis* genome as being orthologs of *E. coli fadD. E. coli* FadD is an acyl-CoA synthase that adds a CoA moiety to free fatty acids, the first step in fatty acid β-oxidation. Since *M. tuberculosis* H37Rv shows much higher expression of *fadD33* than does the avirulent H37Ra strain, this gene was inactivated in H37Rv, using the two-step plasmid method, to test the effect of the disruption on pathogenicity (241). Inactivation of *fadD33* caused a complex virulence phenotype in mice, since the wild-type parent and *fadD33* mutant grew normally in the spleens and lungs of infected mice but the mutant grew less well in livers (approximately 1 log unit less). This phenotype was similar to that exhibited by *M. tuberculosis* H37Ra, and complementation of this latter strain as well as the H37Rv *fadD33* mutant with the wild-type *fadD33* gene restored growth in livers to normal levels. The reason for the tissue-specific phenotype of the mutant is not known, and neither is the actual function of the FadD33 protein.

Other annotated fatty acid metabolic genes (*fadA4*, *fadA5*, and *echA19*) have been identified as being induced in human macrophages by using a promoter trap selection, but only the *fadA4* results were tested and validated by mRNA determination (75). Similar promoter trap experiments have now identified nine annotated *fad* genes as being induced in the lungs of *M. tuberculosis*-infected mice, including *fadA4* (E. Dubnau et al. unpublished experiments). These mouse results have not been validated yet, and no mutations have been made in any promoter trap-identified genes to determine their virulence phenotypes, but these experiments are under way.

(iv) Phospholipases C (Rv2351c, Rv2350c, Rv2349c, Rv1755c, *plcA*, *plcB*, *plcC*, *plcD*). The *M. tuberculosis* genome has four ORFs annotated as encoding phospholipase C-type enzymes. Three of these, *plcA*, *plcB*, and *plcC*, are closely linked to each other, but *plcD* is not. *plcD* is missing or disrupted in many *M. tuberculosis* strains, including H37Rv, and the *plcABC* cluster is not present in *M. bovis* and its BCG derivatives (236). Disruptions of the *plc* genes were obtained by screening a transposon mutant library made in *M. tuberculosis* 103 that has an intact *plcD* gene. In addition, some mutations were made by a two-step plasmid procedure (236). Phospholipase C activity was determined in cell extracts of strains that had individual and multiply mutated *plc* genes, and all individual mutants have lower enzyme activities than that of the wild-type *M. tuberculosis* 103. Triple (*plcABC*) and quadruple (*plcABCD*) mutants have negligible enzyme activity, and strains made by using a *plcABC* mutant as the recipient for individual *plc* genes showed that all restore some activity. *plcABC* in *M. tuberculosis* H37Rv are induced in human (THP-1) macrophages, but the triple and quadruple *plc* mutants of *M. tuberculosis* 103 grow normally in these cells. However, both of these multiple *plc* mutants are attenuated in mice, showing a GIV phenotype.

(v) PanC/PanD (Rv3602c, Rv3601c, *panC*, *panD*). Pantothenate is essential for for the synthesis of CoA and other important molecules involved in fatty acid biosynthesis and degradation, intermediary metabolism, and other cellular processes. The *M. tuberculosis* H37Rv *panC* and *panD* genes, encoding pantothenate synthetase and aspartate-1-decarboxylase, respectively, which are involved in pantothenate biosynthesis, were deleted using the specialized transduction method. The mutant was attenuated for virulence, as determined by measuring the survival time of SCID and immunocompetent mice and the bacterial burden in immunocompetent animals (249). In the former experiments, the SCID mice infected with the *panCD* mutant survived for an average of 250 days while mice infected with the wild-type H37Rv lived an average of 5 weeks. Similar results were observed for immunocompetent mice. Bacterial burden assays showed that the mutant had a PER phenotype in lungs and an SGIV phenotype in spleens and livers. In addition, the histopathologic changes in the lungs of mice infected with the mutant were much smaller than those observed in wild-type infections. Complementation of the mutant with the *M. tuberculosis panCD* genes restored all virulence phenotypes to normal levels. When injected into mice, the mutant was also protective against an aerosol challenge with virulent *M. tuberculosis*, showing protection similar to that conferred by *M. bovis* BCG.

Amino acid and purine biosynthetic genes. As discussed earlier in this section, initial attempts to isolate attenuated *M. tuberculosis* strains focused on amino acid auxotrophs, following a strategy that was used to create effective live attenuated vaccine strains of *S. enterica* serovar Typhimurium (130, 278).

(i) LeuD (Rv2987c, *leuD*). *leuD*, encoding isopropylmalate isomerase, an enzyme that functions in the biosynthesis of leucine, was inactivated by a two-step plasmid procedure in *M. tuberculosis* H37Rv, and it was found that the mutant could not grow in primary murine macrophages or kill SCID mice (131). The *leuD* auxotroph was also able to protect against virulent *M. tuberculosis* infection of wild-type mice to approximately the same extent as *M. bovis* BCG could. *leuD* mutations were also made earlier in *M. bovis* BCG, and the mutants also could not grow in mice, exhibiting a SGIV phenotype (186), or in human macrophages (12).

(ii) TrpD (Rv2192c, *trpD*). TrpD is anthranilate phosphoribosyl transferase, which is involved in the tryptophan biosynthetic pathway. The *M. tuberculosis* gene was inactivated by a two-step plasmid procedure but with an initial denaturing DNA step to increase the frequency of homologous recombination (209). The mutant was severely attenuated in murine macrophages, hardly grew in SCID mice (suggesting a SGIV phenotype), and did not kill any of these mice (266).

(iii) ProC (Rv0500, *proC*). ProC is a pyrroline-5-carboxlate reductase, which is involved in proline biosynthesis, and the *M. tuberculosis* gene was inactivated in the same manner as the *trpD* gene described above (266). Its virulence phenotype is intermediate between that of the the wild-type *M. tuberculosis* H37 parent and the *trpD* mutant (266). It is killed in murine macrophages but not as rapidly as the *trpD* strain, and it kills SCID mice with median killing time of 130 days, in contrast to the wild-type infection, in which all mice are killed by 29 days.

(iv) PurC (Rv0780, *purC*). PurC is 1-phosphoribosylaminoimidazole-succinocarboxamide synthase, which is involved in purine biosynthesis, and it was inactivated in both *M. bovis* BCG and *M. tuberculosis* 103 by a two-step plasmid procedure (141). The growth of both mutants in inactivated murine macrophages is attenuated, with the *M. bovis* BCG mutant showing a large decrease in bacterial numbers while the *M. tuberculosis* mutant does not grow, maintaining the initial numbers of bacteria. Both mutants are severely attenuated in mice with an SGIV phenotype.

Metal uptake. Magnesium and iron are essential for life, and defects in the uptake of these elements frequently lessen the virulence of bacterial pathogens. Following this rationale, two mutants have been made in *M. tuberculosis* that reportedly affect the uptake of these metals and cause the attenuation of virulence.

(i) MgtC (Rv1811, *mgtC*). The *Salmonella* MgtC is a transporter involved in Mg^{2+} uptake (195) and is essential for this pathogen to grow in low-Mg^{2+} media and in macrophages, indicating that this environment is limiting for this divalent cation (117). Since there is an *mgtC* ortholog annotated in the *M. tuberculosis* genome, this gene was inactivated in *M. tuberculosis* Erdman, using a linear DNA construct containing the *M. tuberculosis* Erdman *mgtC* disrupted with an antibiotic resistance cassette (41). The mutant grows poorly in low-Mg^{2+} media and in human MDMs, suggesting that *M. tuberculosis* MgtC has the same function as *Salmonella* PhoP and that the mycobacterial phagosome is also limiting for Mg^{2+}. The mutant is also severely attenuated for growth in mice, exhibiting a SGIV phenotype. However, a similar *mgtC* mutant made in *M. tuberculosis* H37Rv has no attenuated phenotype in THP-1 human macrophages and is able to grow in media containing low Mg^{2+} (S. Walters and I. Smith, unpublished results). The reasons for these discordant results in two laboratories are not known, but different *M. tuberculosis* strains and macrophages were used in the two sets of experiments. The actual role of *mgtC* in *M. tuberculosis* physiology and virulence as well as its regulation awaits further investigation.

(ii) MbtB (Rv2383c, *mbtB*). The *mbt* operon, consisting of *mbtA* through *mbtJ*, encodes enzymes whose function is to synthesize mycobactin and carboxymycobactin (72, 229), the major siderophores in *M. tuberculosis* (235). This regulon is repressed by IdeR in high-iron conditions (110, 244). MbtB is an enzyme in this pathway and catalyzes the formation of an amide bond between salicylate and serine, a step in mycobactin synthesis. Since iron is essential for most lifeforms but is usually in the form of the largely insoluble ferric salts in the environment, iron uptake systems are required to solubilize these salts and to transport the iron into the cell. In bacteria, siderophores usually perform this chelation and solubilization function, and the iron they carry is taken into cells by high-affinity transporters. In addition, pathogens require iron acquisition systems, usually siderophores, during infection to obtain iron from host iron-containing proteins such as transferrin and lactoferrin. In response to infection, the host frequently sequesters iron to prevent bacterial growth (166). Since mutations in siderophore-biosynthetic genes frequently cause attenuation of virulence in bacterial pathogens, the *M. tuberculosis* mycobactin locus was disrupted by inactivation of the *mbtB* gene, using a two-step plasmid procedure (72). The mutant shows wild-type growth in iron-rich media, grows poorly when iron is limiting, and is unable to synthesize the two mycobactin-derived siderophores. It also grows more slowly than the wild type in human macrophages, as measured by a luminescence assay, indicating the mycophagosome may be low in iron. This latter hypothesis is supported by experiments which show that levels of *mbtB* and *mbtI* mRNA are increased during *M. tuberculosis* infection of human macrophages (110). There is no published report on the growth phenotype of the *mbtB* mutant in mice, but several lines of evidence, in addition to its macrophage growth phenotype, suggest that it will be attenuated. These all indicate that iron is limiting in the *M. tuberculosis*-infected host. (i) Excess iron exacerbates the progression of TB in humans and animal models (167). (ii) Measurements of bacterial mRNA in *M. tuberculosis*-infected mouse lungs show that *mbtB* is highly induced compared to its levels when *M. tuberculosis* is grown in broth culture (J. Timm et al., unpublished experiments). (iii) A mutation in *M. tuberculosis* that prevents growth in low-iron-containing media and is thought to affect a component of the high-affinity iron uptake system (244) also severely attenuates growth in mice since the mutant does not replicate; i.e., it has a SGIV phenotype (M. Rodriguez and I. Smith, unpublished results). The identity of this gene is currently under investigation and, when found, should be an important tool with which to study *M. tuberculosis* virulence. Another reason suggesting that iron is limiting for *M. tuberculosis* growth during mouse infections is presented in the following section.

(iii) IdeR (Rv2711, *ideR*). IdeR is a DNA binding protein whose ability to interact with a conserved DNA sequence requires the binding of Fe^{2+} or related divalent cations, and it is a structural and functional homolog of the *C. diphtheriae* DtxR (223, 255). IdeR is the major mycobacterial regulator of iron uptake and storage genes, repressing the former and activating the latter (79, 110, 243, 244). *ideR* is an essential gene in *M. tuberculosis* and can be inactivated only in the presence of a second site suppressor (244); thus, it has not been possible to directly assess the role of IdeR in virulence. However, it is included in this section on virulence factors because of a report that the presence of a mutated DtxR, which exhibits iron-independent repression in other contexts, decreases the growth of *M. tuberculosis* in mice (176). Although it was not demonstrated directly in vitro or during infection, it was postulated that the mutated DtxR is repressing *M. tuberculosis* iron uptake genes during growth of the bacterium in mice. Despite certain caveats, this interesting observation suggests that iron acquisition is essential for *M. tuberculosis* growth in mice and could be a target for therapeutic intervention, as discussed below.

Anaerobic respiration and oxidative stress proteins. *M. tuberculosis* was originally thought to be an obligate aerobe, but much work referred to in this review indicates that it may encounter microareophilic environments during later stages of infection, e.g., in lung granulomas. On the other hand, most aerobic organisms, including bacteria, have enzymes that degrade peroxides and H_2O_2, which are normal by-products of aerobic respiration and can give rise to toxic ROIs if allowed to accumulate. These enzymes, generally superoxide dismutases and catalases, as well as related enzymes, are also important for the response to various external oxidative stresses. Since phagocytic cells produce ROIs to kill invading bacteria, it is not surprising that these enzymes are important for *M. tuberculosis* virulence.

(i) Nitrate reductase (Rv1161, *narG*). NarG is a subunit of the prokaryotic respiratory (anaerobic) nitrate reductase that plays major role in respiration in the absence of oxygen, and anaerobic nitrate reductase activity increases when *M. tuberculosis* becomes microaerophilic (306). The *M. tuberculosis* genome has several ORFs with similarity to genes encoding the subunits of nitrate reductase, including one gene cluster that is annotated as *narGHIJ*, the usual nitrate reductase-encoding

genomic structure in prokaryotes (53). The *M. tuberculosis narGHIJ* cluster was shown to encode anaerobic nitrate reductase proteins, and a *narG* mutation was made in *M. bovis* BCG by using a two-step plasmid procedure (309). The mutant had no anaerobic nitrate reductase activity, but its growth under aerobic or anaerobic conditions was unaffected. When the *M. bovis* BCG *narG* mutant was used to infect mice, a significant virulence phenotype was observed. When SCID mice were infected, the wild-type parent grew well while the mutant showed no replication but was not cleared. In normal mice, the wild-type BCG strain did not replicate but the mutant was rapidly cleared from the lungs, livers, and kidneys, exhibiting a SGIV phenotype (102). These results have not been confirmed for *M. tuberculosis* yet, but they suggest that anaerobic or microaereophilic growth is an important feature of *M. tuberculosis* physiology during infection.

(ii) KatG (Rv1908c, *katG*). KatG is a catalase:peroxidase that degrades H_2O_2 and organic peroxides. It is the only enzyme with catalase activity in *M. tuberculosis*, and in addition to degrading ROIs, it activates the prodrug INH to form a reactive species that inhibits mycolic acid biosynthesis (183). Spontaneous mutations to INH resistance are usually found in *katG*, and a mutant of this type in *M. bovis* was attenuated in guinea pigs in a spleen morbidity assay (317). In addition, an *M. tuberculosis* H37Rv *katG* mutant, also isolated on the basis of its resistance to INH, was shown to be attenuated in the lungs and spleens of infected mice and was rapidly cleared after initial normal growth, a PER phenotype (125). Another *katG* mutant of *M. tuberculosis* H37Rv showed a similar PER-type phenotype in mice, and virulence in guinea pigs was also attenuated by the *M. tuberculosis katG* mutation. Complementation with the wild-type *katG* restored enzyme activity and virulence (163). In *M. smegmatis* (328) and *M. tuberculosis* (228) *katG* is negatively regulated by the FurA protein, whose structural gene is directly upstream of *katG*. The role of FurA in virulence is not currently known.

(iii) AhpC (Rv2428, *ahpC*). AhpC is an alkyl hydroperoxide reductase, and enzymes of this type function to detoxify organic hydroxyperoxides. Attempts to inactivate this gene in the *M. tuberculosis* complex have been unsuccessful, and an antisense method was used to phenotypically lower the expression of the *ahpC* gene in *M. bovis* (315). The resulting phenotypic mutant produced less AhpC than did the wild type and was more sensitive to H_2O_2 and cumene hydroperoxide. The mutant was also much less virulent in a guinea pig model, showing 3 log units fewer CFUs than the wild type. It has been postulated that AhpC can compensate for the lack of lack of catalase:peroxidase activity in *M. tuberculosis katG* mutants, since there some reports of increased *ahpC* expression in some *katG* mutants (125, 262). However, levels of AhpC are not correlated with the virulence of *katG* mutants (125).

(iv) SodA (Rv3846, *soda*). SodA is the iron-factored superoxide dismutase that degrades superoxides, which are normal by-products of normal aerobic respiration and are also produced by the phagocytic repiratory burst enzyme. It is therefore important for the survival of intracellular pathogens during infections. SodA is the major enzyme with this activity in *M. tuberculosis*, and attempts to inactivate this gene have not been successful (81), unlike the situation in *M. smegmatis*, where it was possible to inactivate *sodA* (79). To circumvent this problem, as was done with the *ahpC* gene discussed above, an antisense approach was used to make a phenotypic *sodA* mutation in *M. tuberculosis* H37Rv (82). The phenotypic mutant produced much less SodA protein and was severely attenuated in mice, showing up to 5 log units fewer CFUs than the wild type in lungs and spleens, and it was rapidly cleared, demonstrating an SGIV phenotype. Recent results have indicated that *M. tuberculosis* SodA inhibits redox signaling by macrophages, and it is proposed that this would affect the initiation of the cellular immune response after infection (D. Kernodle, personal communication).

(v) SodC (Rv0342, *sodC*). SodC is the Cu,Zn-factored superoxide dismutase that is responsible for a small part of total Sod activity in *M. tuberculosis*. Two laboratories have inactivated this gene in *M. tuberculosis*, with different virulence results. In one case, *sodC* was inactivated in *M. tuberculosis* Erdman, using a linear DNA construct, and the resulting mutant was more sensitive to superoxides than the wild type and was killed more efficiently than the wild-type parent in activated primary (peritoneal) murine macrophages. It was not affected in inactivated murine macrophages or activated macrophages from respiratory burst-deficient mice (222). In the other study, the *M. tuberculosis* H37Rv *sodC* was inactivated by a two-step plasmid procedure, and while this mutant also showed increased sensitivity to superoxides and H_2O_2, it exhibited wild-type growth in activated primary (bone marrow) murine macrophages and in guinea pigs (81). The reasons for the discrepant results are not known, but different *M. tuberculosis* strains and macrophages were used.

Transcriptional Regulators

Since transcriptional regulators control the transcription of many genes, a directed mutational strategy to inactivate regulatory genes would be expected to find some that are important for *M. tuberculosis* virulence, as has been demonstrated in other pathogens, such as the *S. enterica* serovar Typhimurium virulence factors alternative sigma factor RpoS (88) and the response regulator PhoP (92, 192).

Sigma factors. One of the major strategies used by prokaryotes to radically change their life-style in response to a changed environment involves using RNA polymerase holoenzymes with different promoter specificities. This is achieved by the formation of new holoenzymes containing different sigma factors, which allows the transcription of genes required for the new conditions. Pathogens, including *M. tuberculosis*, also use this tactic for the transcription of genes that are important for virulence.

(i) Sigma A (Rv2703, *sigA*). Sigma A is the essential principal mycobacterial sigma factor and is presumably necessary for most mycobacterial housekeeping gene transcription (112, 225). Unlike the products of most of the other transcriptional regulatory genes that were directly inactivated, sigma A was identified as a virulence factor by complementation of an attenuated *M. bovis* strain (ATCC 35721) with an *M. tuberculosis* cosmid library, using a guinea pig morbidity assay (56). The original mutation in *sigA* that causes attenuation is a partial loss of function that allows the sigma A protein to function in general transcription, since the mutant grows normally in vitro (broth cultures and solid media), but it is presumably unable to

transcribe at least one virulence gene. The attenuating mutation is an arginine-to-histidine change at amino acid residue 515 (R515H) of the protein and is localized to a C-terminal domain that, in other sigma factors, interacts with transcriptional activators. This suggested that sigma A must interact with a transcriptional activator that allowed the expression of a gene(s) necessary for virulence (112). Recent experiments have confirmed this hypothesis since it has been shown that WhiB3 (Rv3416) interacts with sigma A (277). This important finding is discussed below. Hopefully, DNA array analyses will soon compare the global expression profiles of strain ATCC 35721 and its complemented derivative strain to see which genes are not transcribed in the mutant strain, as has been done for other *M. tuberculosis* sigma factor mutations. These analyses should allow the identification of genes in the sigma A regulon that require WhiB3 and should ultimately lead to the identification of those that are essential for virulence.

(ii) Sigma F (Rv3286c, *sigF*). The derived amino acid sequence of *M. tuberculosis* sigma F is very similar to those of the sigma F proteins of *S. coelicolor* and *B. subtilis*, which are essential for sporulation in these two species, as well as to those of sigma B of *B. subtilis*, which controls responses to environmental stress (68). It was speculated that the latency of *M. tuberculosis* in human TB could be similar to bacterial sporulation, and to provide evidence for this hypothesis, *sigF* in *M. tuberculosis* CDC1551 was inactivated by allelic replacement, using a two-step plasmid method (52). The mutant has no macrophage phenotype and is attenuated for virulence in mice, using mortality as a criterion. The mice infected with the mutant all died by 334 days after infection (50% died at 246 days), while mice infected with the wild type were all dead by 184 days (50% died at 161 days). It is not known which genes transcribed by RNA polymerase containing sigma F (RNAP-sigma F) are important for this virulence phenotype. Recently, direct transcription assays have identified genes transcribed by RNAP-sigma F, and a promoter sequence has been identified that strongly resembles the *B. subtilis* RNAP-sigma B consensus promoter sequence (19). This information and future DNA array analyses should allow the identification of sigma F-dependent *M. tuberculosis* virulence genes. Interestingly, this latter work also showed that the activity of sigma F is controlled posttranslationally by its binding to an anti-sigma protein (Rv3287c) that previously had been identified as having sequence similarity to the anti-sigma F and anti-sigma B proteins of *B. subtilis* (67). In turn, the activity of the *M. tuberculosis* anti-sigma F protein is downregulated by its binding to two different anti-anti-sigma factors, Rv1356c and Rv3687c, an interaction that allows sigma F to function (19). Significantly, the function of Rv1356c is regulated by the redox potential, while it is proposed that Rv3687c activity is controlled by phosphorylation (19)

(iii) Sigma E (Rv1221, *sigE*). Sigma E is a member of the ECF (for "extracytoplasmic function") group of sigma factors that control the bacterial response to external stimuli. *sigE* transcripts are induced after exposure of *M. tuberculosis* to various environmental stresses such as high temperature and detergent stress (179). Since these stresses might be found during *M. tuberculosis* infections and since hybridization-based methods showed that *sigE* mRNA levels increased during *M. tuberculosis* growth in human macrophages (115, 144), there was a possibility that sigma E would be necessary for virulence. To test this idea, *sigE* was inactivated in *M. tuberculosis* H37Rv by allelic replacement, using a two-step plasmid procedure, and its phenotype was analyzed in vitro and during infection of mouse and human macrophages (182). The mutant is more sensitive to detergent, high temperature, and oxidative stress than is the wild-type parent *M. tuberculosis* H37Rv and grows more poorly than the wild type in both types of macrophages. Preliminary results show that the mutant is attenuated in wild-type mice with a GIV phenotype and kills SCID mice more slowly than the wild type does: all mice infected with the mutant were dead by 70 days, while *M. tuberculosis* H37Rv killed all mice by 30 days (R. Manganelli et al., unpublished results). DNA array analyses comparing the *sigE* mutant and the wild-type parent showed that 38 genes required sigma E for their expression during normal growth while 23 other genes in 13 transcription units required this transcription factor for their induction after sodium dodecyl sulfate (SDS) stress. Nine of these transcription units had a conserved ECF sigma factor-like promoter sequence in the region directly upstream of the first gene in each unit. Among the genes requiring sigma E for their expression during unstressed growth are some encoding proteins involved in translation, transcriptional control mycolic acid biosynthesis, electron transport and the oxidative stress response. Genes requiring sigma E during SDS stress encode proteins that are involved in fatty acid degradation, some that are heat shock proteins, and several that are putative transcriptional regulators. *sigB* encoding sigma B, a nonessential sigma factor (M. Gomez and I. Smith, unpublished results), required sigma E under both stressed and unstressed conditions, and recent experiments have shown that RNAP-sigma E can transcribe *sigB* (Rodrigue et al., unpublished). *sigB* mutations do not affect *M. tuberculosis* pathogenicity (Gomez and Smith, unpublished), nor is it known which of the other genes requiring sigma E for their transcription are necessary for virulence. This question is currently being investigated. As is the case with many ECF sigma factors, sigma E activity is downregulated by an anti-sigma factor, RseA, that is encoded by a gene, Rv1222, adjacent to *sigE* (Rodrigue et al. unpublished). The possible role of RseA in virulence is not known and is also being studied.

(iv) Sigma H (Rv3223c, *sigH*). Sigma H is another member of the ECF family of sigma factors, like sigma E, and is very similar to the sigma R of *Streptomyces* species. Sigma R responds to certain types of oxidative stress, such as diamide treatment, that oxidize protein-SH groups, which then form intramolecular disulfide bonds (207). Its promoter recognition activity is blocked by binding of an anti-sigma factor, RsrA, that is encoded by a gene adjacent to *sigR*. On oxidative (diamide) stress, key SH groups in RsrA become oxidized and its binding to sigma R is disrupted (150). Sigma R is then able to transcribe several genes such as its own structural gene *sigR* and the *trx* operon, encoding thioredoxin and thioredoxin reductase, which can reduce proteins that were oxidized by diamide treatment. Thioredoxin and its reductase function to return the system to the unstressed state, since the newly reduced RsrA can again bind to sigma R (206). The *M. tuberculosis sigH* is induced after various stresses like heat shock and SDS treatment (179) and during macrophage infection (115). Subsequently, this gene was inactivated in three laboratories

(151, 181, 232). The phenotype of the *sigH* mutant is as expected from the *Streptomyces* experiments and the earlier *M. tuberculosis* gene expression experiments, since it is sensitive to SDS, diamide, and heat shock. A combination of DNA array analyses and individual gene expression assays showed that there are no genes that require sigma H during unstressed growth (181), but genes similar to those transcribed by the *Streptomyces* RNAP-sigma R, including those encoding thioredoxin reductase and two thioredoxins, are dependent on sigma H for their expression after diamide stress (151, 181, 232). Many of these *M. tuberculosis* genes have a promoter sequence identical to the one used by the *Streptomyces* RNAP-sigma R and also the one thought to be recognized by mycobacterial RNAP-sigma E. Transcription assays have shown that some of these genes are transcribed by mycobacterial RNAP-sigma H (232). Among these is *sigB*, which is also transcribed by mycobacterial RNAP-sigma E which uses the same *sigB* promoter as the RNAP-sigma H (Rodrigue et al, unpublished). The virulence phenotype of the *sigH* mutant is subtle in that its growth in macrophages and mice is normal in terms of bacterial load (151, 181), but there are differences in lung histopathology, including fewer granulomas and a generally delayed pulmonary inflammatory response (151). As is the case for *Streptomyces*, *M. tuberculosis* has an anti-sigma H factor, RshA (Rv3221A), whose sequence is similar to that of the *Streptomyces* RsrA, and its structural gene maps near *sigH*. Biochemical experiments have shown that the purified *M. tuberculosis* RshA binds to sigma H, preventing it from functioning in transcription, similar to the *Streptomyces* RsrA-sigma R interaction (Rodrigue et al., unpublished).

Response regulators. The other major strategy used by bacteria to respond to changing environments involves elaborate signal transduction networks, These are the "two-component systems" that respond to environmental signals via sensor proteins, histidine kinases, which, in turn, activate cognate effector proteins, the response regulators, which are usually transcriptional regulatory factors. As discussed earlier in this review, bacteria have multiple two-component systems, each responding to different stimuli, and several laboratories have made mutations in *M. tuberculosis* two-component genes to test their effects on virulence.

(i) PhoP (Rv0757, *phoP*). PhoP shows high similarity to the PhoP response regulator of *S. enteric serovar* Typhimurium, which senses Mg^{2+} starvation and controls the expression of virulence genes (117). On this basis, *phoP* was disrupted in a clinical *M. tuberculosis* isolate, strain MT103, by a two step plasmid procedure and virulence phenotypes were determined (219). The mutant grows poorly in mouse macrophages and is severely attenuated in mouse organs, where it has an SGIV phenotype. These results have been confirmed and extended as *phoP* has been disrupted in *M. tuberculosis* H37Rv by the two-step plasmid procedure and this mutant is also attenuated in human and mouse macrophages as well as in mice, where it is has a SGIV phenotype (Walters and Smith, unpublished). In vitro experiments have shown that the *M. tuberculosis* H37Rv *phoP* mutant grows poorly in low-Mg^{2+}-containing media, and it is now thought that the *M. tuberculosis* PhoP senses Mg^{2+} starvation as does the *S. enteric* serovar Typhimurium PhoP (Walters and Smith, unpublished). Genes controlled by PhoP are not known yet, including those important for virulence, and this is currently being investigated.

(ii) PrrA (Rv0903c, *prrA*). PrrA is one of the 13 annotated response regulators in the *M. tuberculosis* genome. It had been previously shown that this gene was upregulated during *M. tuberculosis* infection of human macrophages (115), and screening of an ordered transposon mutagenesis library for mutants found one with an insertion near the beginning of the coding sequence for PrrA (86). The growth of the *prrA* mutant in mouse primary macrophages was slightly lower than that of the wild-type *M. tuberculosis* 103 at 3 and 6 days, reaching wild-type levels at 7 days, and the mutant grew essentially as well as the wild type in mice, with a small decrease in bacterial load that was observed only in spleens. In agreement with these observations, the use of GFP reporter fusions with the *prrA* promoter showed that this gene was transiently induced in macrophages, with peak levels of gene expression 4 h after infection and with levels declining after that time. The significance of PrrA to virulence is not clear, given the very subtle attenuation phenotype.

(iii) Rv0981 (Rv0981, *mprA*). Rv0981 is another *M. tuberculosis* two-component response regulator, and it was inactivated in *M. tuberculosis* H37Rv by a two-step plasmid procedure (327). The mutant had an unusal phenotype in that it grew better than the wild type in murine macrophages and human MDMs. However, it did not persist in the lungs and livers of infected mice, growing initially and being cleared after 140 days, respectively, showing a delayed PER phenotype. *mprA* is induced when *M. bovis* BCG infects macrophages, using a *mprA*-GFP fusion, but the same *mprA*-GFP construct in *M. tuberculosis* H37Rv does not show induction of *mrpA* in macrophages (327).

Virulence phenotypes of other two-component gene mutations have been measured, e.g., in the reponse regulator RegX3 (Rv0491) and in the histidine kinase TrcS (Rv1032c). No macrophage phenotype was observed, and these genes were not induced in macrophages (86). In agreement with these results, 10 of the 13 two-component response regulator genes in *M. tuberculosis* were inactivated and only the *phoP* mutant (discussed above) had a virulence phenotype in human macrophages (Walters and Smith, unpublished). Included in this group were mutants with disruptions in *regX* and *trcS*. Other two-component systems in *M. tuberculosis* have been studied. MtrA-MtrB (Rv3264c-Rv3245c) is essential for growth since *mtrA* cannot be disrupted (326). Interestingly, GFP reporter fusions with the *mtrA* promoter in *M. bovis* BCG are induced in murine macrophages, but this does not occur when the fusion is in *M. tuberculosis*, as was observed in gene expression studies with Rv0981 (discussed above). Another response regulator, DosR (Rv3133c), controls the global response to oxidative stress and low oxygen response, including the expression of the anoxia-induced *hspX* gene as well as its own expression (263). A proteomic analysis of *M. bovis* BCG grown under microaerophilic conditions showed upregulation of the same genes (34); *M. smegmatis* genes corresponding to Rv3133c and Rv3132c, encoding the cognate histidine kinase and *hspX*, were also induced in anoxic conditions (185). Thus, DosR is important for the mycobacterial response to various environmental stresses, and a recent report shows that disruption of the *M. bovis* BCG *dosR* causes a loss of viability during

long-term hypoxia, using an vitro model for *M. tuberculosis* persistence (33). While a mutation in *M. tuberculosis* Rv3133c has no effect on *M. tuberculosis* growth in human macrophages (Walters and Smith, unpublished), it would be expected to cause an attenuated phenotype in mice, given its effect on bacterial survival in vitro, but this has not get been tested.

Other transcriptional regulators. In addition to sigma factors and response regulators, bacteria use other types of transcriptional regulators to control the expression of large groups of genes. As discussed above, there are many ORFs that are annotated as transcriptional regulators in the *M. tuberculosis* genome, but surprisingly few have been studied to determine their functions in general physiology and virulence. Among the rarely characterized *M. tuberculosis* regulatory proteins that affect virulence are IdeR (see above), as well as HspR and WhiB3. Other transcriptional regulators have been described that are potentially important for virulence, like RelA (226), since *relA* mutant shows defects in long-term survival in vitro while its macrophage growth is normal. However, there have been no published reports describing the *relA* mutant phenotype in animal models.

(i) HspR (Rv0353, *hspR*). HspR is a repressor of key heat shock genes like *hsp70*, where it binds to a specific DNA sequence, the HAIR element (for "HspR-associated inverted repeat") in the *hsp70* promoter region, in *S. coelicolor* (40) and *H. pylori* (272). The repression occurs at the permissive temperature of 37°C and is lifted at 45°C. *M. tuberculosis* also has an ortholog of this ORF (113). The synthesis of *M. tuberculosis* heat shock proteins, some of which are immunodominant antigens (322), is increased after infection (159). Investigations were carried out to see whether the *M. tuberculosis* HspR was a repressor of *hsp70* and whether the regulation of heat shock proteins would be important for virulence (275). Biochemical studies showed that purified *M. tuberculosis* HspR binds to DNA sequences containing the HAIR element, and physiological experiments indicated that *hspR* mutants in both *M. tuberculosis* and *M. bovis* BCG, constructed by the two-step plasmid technique, are derepressed for Hsp70 synthesis at 37°C, unlike the wild-type strains. The *M. tuberculosis* mutant survives better than the wild type after heat shock, presumably because of the protective effect of the higher level of HSPs. Thus, *M. tuberculosis* HspR functions in the same manner as other bacterial HspRs. While the *M. tuberculosis hspR* mutant has no phenotype in murine macrophages, it is attenuated in mice, with a GIV phenotype. The reason for this attenuation is not known, but it was suggested that the higher levels of HSPs in the mutant may cause increased host immunosurveillence followed by more efficient killing of the pathogen (275).

In addition to potential roles in immunosurveillance, *M. tuberculosis* HSPs may play a more direct role in virulence. GroES is a highly conserved HSP that has chaperonin activity and is also known as cpn10. *M. tuberculosis* GroES (Rv3418c) is found as a major constituent in the culture filtrate or media in which *M. tuberculosis* grows (271), suggesting that it will be directly exposed to the intraphagosomal milieu. Recombinant *M. tuberculosis* GroES is a stimulator of bone resorption and induces osteoclast recruitment in bone explant cultures while also inhibiting the proliferation of an osteoblast bone-forming cell line (189). It has recently been shown that *M. tuberculosis* GroES binds calcium (284). This suggests a role for GroES in Pott's disease, the extrapulmonary form of TB discussed earlier in this review that is marked by the weakening and resorption of spinal vertebrae. This role could be related to the physical depletion of calcium in bones and/or the disruption of calcium signaling in host cells by GroES during-long term TB infection (284).

(ii) WhiB3 (Rv3416, *whiB3*). WhiB was originally described in *S. coelicolor*, and disruptions of its structural gene and that of WhiD, a closely protein, prevent sporulation and cell septation in this bacterium (193). There are seven members of the WhiB family in *M. tuberculosis* H37Rv, and it has been speculated that the persistence or latent state of *M. tuberculosis* is analagous to bacterial sporulation (270). For this reason, studies of mycobacterial WhiB orthologs have been performed, initially with *M. smegmatis*. It was shown that WhmD, a WhiB2 homolog in *M. smegmatis*, is required for cell division and septation (111) but that a disruption of the *M. smegmatis whiB3* homolog had no effect on cell growth or stationary-phase survival (139). In *M. tuberculosis*, as previously mentioned, a search utilizing a yeast two-hybrid system has found that *M. tuberculosis* WhiB3 specifically interacts with the region 4.2 domain of the wild-type *M. tuberculosis* sigma A but not the equivalent region from the protein containing the R515H mutation (277). *whiB3* was inactivated in *M. tuberculosis* H37Rv, and while there was no effect on bacterial growth in either mice or guinea pigs, mice infected with the mutant survived longer than did those infected with the wild-type parent; i.e., mice infected with the wild type all died by 225 days, with 50% of the animals dying at 150 days, and the comparable figures for mouse infections with the *whiB3* mutant were 350 and 260 days. The same *whiB3* mutation was made in *M. bovis* and resulted in a mutant that was severely attenuated for growth in guinea pigs, exhibiting a bacterial load in spleens that was 5 log units lower than that of the wild-type *M. bovis* (277). This attenuation phenotype is similar to that observed in the original *M. bovis sigA* R515H mutant (56). This result indicates significant differences in virulence mechanisms of these two members of the *M. tuberculosis* complex. Genome comparisons of *M. tuberculosis* and *M. bovis* have shown that the former species has 61 ORFs that are not found not in the latter (20), but the role of most of these proteins (outside of those in the RD1 region) in virulence or the dependence of their expression on sigma A and WhiB3 is not known. Also not known is the role in virulence of other members of the *M. tuberculosis* WhiB family. A promoter trap search has identified *whiB2* (Rv3260c) as being differentially expressed in the lungs of *M. tuberculosis* H37Rv-infected mice, and the possible function of this protein in virulence is currently being studied (Dubnau and Smith, unpublished).

FUTURE RESEARCH

Genetic Approaches

As shown in this review, much work has been performed in the search for *M. tuberculosis* virulence factors. Various targets have been identified, e.g., enzymes involved in the synthesis of unique cell wall structures and secreted proteins. More work has to be performed so that that every potential gene is systematically inactivated in the *M. tuberculosis* genome and the

virulence of these mutants can be assayed. Ideally, the mariner transposon system (253) can be modified to incorporate signature tagging so that many potential mutants can be tested in vivo at the same time. In addition to this global search, there are more specific approaches that can be undertaken. For example, as mentioned earlier in this review review, some *M. tuberculosis* clinical strains are more virulent than others (178). The numerous comparative genomic sequencing projects being conducted now may identify potential genes that are responsible for these phenotypes, and these could then be individually characterized. As previously discussed, all *M. bovis* BCG strains have one common deletion, RD1, that causes their attenuated phenotype (162, 227). Of the nine genes in the deleted region, only Rv3875, encoding Esat6 has been implicated in virulence, since a mutant with a disruption in this gene is attenuated for virulence (305). It is not known whether any of the other genes in the RD1 region are also important for *M. tuberculosis* pathogenicity, and it should be relatively easy to use knockout strategies to answer this question, which has significance for making a better "BCG-like" live-cell vaccine.

New Ways To Study *M. tuberculosis*-Host Interactions

As an intracellular pathogen, *M. tuberculosis* has developed global strategies to survive and grow in macrophages and granulomas formed in various organs of its host. In the same way, the infected phagocytic cell and surrounding tissue respond in a global sense to the presence of an intruding pathogen. As discussed earlier in this review, DNA arrays have been used to study the global expression of genes in wild-type and mutant *M. tuberculosis* strains grown under different conditions. Proteomic techniques have also been used to measure the levels of large numbers of bacterial proteins when *M. tuberculosis* is in different environments. The purpose of this section is to discuss how new global methods are currently being used and could be used in the near future to study the interactions between *M. tuberculosis* and its host.

***M. tuberculosis*-macrophage interactions.** There have been some descriptions of host responses when macrophages are allowed to phagocytose *M. tuberculosis* or purified bacterial components like LAM. However, these macrophage responses have generally been limited to the expression of a small number of genes or the levels of a few proteins, and as discussed earlier in this review, there are often widely discordant results from different laboratories. e.g., the effects of adding the 19-kDa glycolipoprotein to macrophages. Recently, DNA array analyses have been used to study host gene expression during *M. tuberculosis* infection of human (198) and murine (83) macrophages. The latter study showed that exposure of murine macrophages to IFN-γ and *M. tuberculosis* altered the expression of 25% of the monitored mouse genome (83). This study also used primary macrophages from mutant mice, providing significant information on mechanisms of the host response to infection.

To more completely describe the *M. tuberculosis*-macrophage interaction, the next step will be to combine global expression methods to analyze the expression of both bacterial and host genes during the same macrophage infections. As an example of this combined approach, mutations in the *M. tuberculosis* regulatory genes *sigE* (182) and *phoP* (219) (Walters and Smith, unpublished) cause attenuation of virulence phenotypes, and DNA array analyses with both mutants identified several genes that require sigma E or PhoP for their expression. Recent developments in DNA array technology have now made it feasible to use much smaller amounts of bacterial RNA (1 to 2 μg) for these studies. Thus, global expression profiling with DNA arrays can now be used to identify the *M. tuberculosis* genes that require sigma E or PhoP during the infection of macrophages, i.e., comparing the expression profile of the *sigE* or *phoP* mutants with that of the wild-type *M. tuberculosis* strain. This was recently done with wild-type *S. anterica* serovar Typhimurium infecting murine macrophages (85). At the same time, it will be possible to globally compare the expression of macrophage genes during the infection with wild-type *M. tuberculosis* and the two regulatory mutants, as was done with *S. enteria* serovar Typhimurium *phoP* mutant and wild-type strains during a human macrophage infection (71). However, since these regulators presumably control many genes that are differentially expressed during macrophage infection, it may not be possible to determine initially the identity of the bacterial effector molecules important for virulence that are missing in the mutants. Thus, it will be important also to characterize the *M. tuberculosis* genes requiring the regulators during macrophage infection by performing functional assays and by testing the virulence phenotypes of mutations in their structural genes. Since mutations in some *M. tuberculosis* effector molecules that affect virulence are already known, e.g., in the 19-kDa protein and other secreted or membrane-associated proteins, it will be important to use these mutants to study global host gene expression. In these experiments, one could compare the effect of the presence or absence of a molecule like the 19-kDa protein on host gene expression during *M. tuberculosis* infection of macrophages. The results of such studies, in which effector molecules are presented to the macrophage in their normal context or are not presented, should clarify their roles in virulence.

For want of a better term, this section discusses *M. tuberculosis*-host interactions at stages after the initial encounter between the bacterium and the macrophage. As discussed above, one of the early stages in progression of TB is the formation of a granuloma as monocytes and lymphocytes are recruited to the site of the original infection in the lung, ultimately surrounding and isolating the infectious bacteria, if the infection is controlled. Thus, it is important to study *M. tuberculosis* virulence at these stages, using the new technologies described in this review. Generally, most experiments have been done in animal models, and at various stages in infection, infected organs, e.g., lungs, livers, and spleens, are isolated along with the bacteria and various assays are performed. There have not been many studies involving systems that are not as complex as the whole infected animal but that allow the interaction between infected macrophages and lymphocytes. Several attempts have been made to bridge the gap between simple ex vivo (macrophage) and in vivo (animal model) experiments, and two examples are cited. A whole-blood assay has been used to study early stages in *M. tuberculosis* infection, and it utilizes blood drawn from TB patients or noninfected individuals (303). The advantages of this system are that monocytes and lymphocytes present in blood can interact as they do ordinarily and that *M. tuberculosis* is rapidly phagocytosed and

remains intracellular for at leat 72 h. Another system assembles cellular components thought to be involved in early *M. tuberculosis* infection, by using a tissue culture bilayer system (28). In this study, human lung epithelial type II pneumocytes were separated from endothelial cells by a microporous membrane and the movement of *M. tuberculosis* and monocytes through the membrane and their interaction was studied. It would seem that systems like these can be used to study bacterial and host responses in a much more physiological setting than in simple macrophage infections.

It is also essential to have more efficient ways to study *M. tuberculosis* in an actual granuloma, since this is where the bacterium spends the majority of its lifespan during an infection. As discussed in this review, some studies of *M. tuberculosis* gene expression in mouse organs have been performed by using RT-PCR techniques to measure the mRNAs of individual genes, but this approach is limited by its individual gene-by-gene nature. A global promoter trap technique has allowed the identification of several genes that are induced in mouse lungs (Dubnau and Smith, unpublished), but this method is quite labor-intensive and takes a long time to first identify and then validate putative upregulated genes. Clearly, a global gene expression method like DNA array analyses is needed. Unfortunately, current DNA array methods are not sensitive enough to use the purified *M. tuberculosis* RNA purified from one infected mouse lung. However, it may be possible to isolate sufficient bacteria from lung granulomas of infected rabbits since these are much larger and more delimited than those of mice and frequently contain large numbers of *M. tuberculosis* (29). In addition, it will be important to analyze host expression in cells comprising the granuloma. A major problem with this type of study, which also must be considered in macrophage response experiments, is the contribution of noninfected cells to any quantitative analysis. This problem is tractable in tissue culture studies, since protocols can be readily designed to ensure that essentially all macrophages are infected, but the problem is much more complex in infected organ tissues. A relatively new technique, laser capture microscopy (LCM), has been developed to isolate pure cells from specific microscopic regions of tissue sections (32). A laser beam focally activates a special transfer film, which binds to cells in the tissue section and allows their isolation from unwanted cells on the basis of desired criteria such as cytochemical and/or immunological staining. The cells and their macromolecules are not affected by the laser beam because its energy is absorbed by the film. One study of *M. tuberculosis* infection in mice has shown that a panel of nine host genes isolated from lung granulomas by LCM show patterns of gene expression that are markedly different from those obtained from whole infected lungs (J. Chan, personal communication). It is expected that LCM will soon be widely used to study the host response in granulomas formed during *M. tuberculosis* infections and will provide much important information.

PROSPECTS FOR NEW ANTITUBERCULAR AGENTS

With several *M. tuberculosis* targets already found and the expectation of finding new ones, the next question is what should be done with this information. It is beyond the scope of this review to discuss in detail new vaccines and drugs, and these areas have been recently reviewed (18, 299). These concluding remarks will list some of the areas that could be exploited for better therapies. It is still worthwhile to pursue live vaccines that are more protective than *M. bovis* BCG, even though the problem of administering them to immunocompromised individuals must be addressed. The use of vaccines involving recombinant *M. bovis* BCG containing *M. tuberculosis* genes is a new approach that has great promise (133), and it will be important to use other *M. tuberculosis* genes in this delivery system as well. Another approach to the synthesis of live vaccines is to systematically use attenuated *M. tuberculosis* mutants for protection studies. In this regard, the protection results obtained with a phenotypic *M. tuberculosis sodA* mutant are extremely encouraging (82). It will be important to use for protection assays other mutants that are obtained by standard allelic replacement since there will be a lower chance of reversion. For this purpose, silent mutations, i.e., those made without antibiotic resistance cassettes, will be necessary. Subunit vaccines and DNA vaccines are other areas that should be developed as more *M. tuberculosis* virulence factors are identified. The identification of new antitubercular drugs is an important and complex undertaking, and again it would seem that unique systems found in *M. tuberculosis* are prime candidates as new targets for rational drug design. One area, among others, that should be exploited is the *M. tuberculosis* iron acquisition system, since this essential element is limiting for *M. tuberculosis* growth in vivo in animal experiments (176, 244). Since *M. tuberculosis* siderophores, and presumably their receptors, are highly specific, it should be possible to use them to deliver toxic agents directly to *M. tuberculosis* without affecting the host.

The advances in our understanding of *M. tuberculosis* virulence summarized in this review are expected to result in many new treatments that should help prevent or control the spread of TB throughout the world. However, it is again important to remember the history of TB research starting over 100 years ago that led to the development of the BCG vaccine and then the discovery of antibiotics over 50 years ago. We must remember Rene Dubos' cautionary words from 1952, mentioned at the beginning of this review, that are still as pertinent as ever (77). They can be restated in 2002 as follows: doctors and researchers must continue their fight against TB, and the new vaccines and drugs that are developed will hopefully be more effective than *M. bovis* BCG, streptomycin, and INH were in the past. However, TB will be completely eradicated only when poverty and unequal development are ended throughout the world.

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