

Deletion of a *Mycobacterium tuberculosis* Proteasomal ATPase Homologue Gene Produces a Slow-Growing Strain That Persists in Host Tissues

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The in vivo rate of proliferation of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, has been linked to the rate of progression and severity of disease. Here, we report that deletion of the gene MT2175 (Rv2115c), a putative mycobacterial proteasome-associated AAA-ATPase, leads to a reduction in the growth rate of *M. tuberculosis* in vitro and in vivo. Despite the reduced growth, the mutant persisted, with slow and gradual clearance in mouse lungs. The mutant elicited reduced levels of interferon- γ production in the lungs and, when used as an immunizing agent, provided significant protection against challenge with a virulent strain of *M. tuberculosis*. Expression of the genes *lat* and MT3159 were highly up-regulated in the mutant. Thus, loss of MT2175 slows both in vitro and in vivo growth rates and compromises the lethality of *M. tuberculosis* in mice but has a minimal impact on the organism's ability to persist in host tissues.

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), is one of the deadliest pathogens known to humans and kills ~2–3 million people each year [1]. The recent emergence of multidrug-resistant strains detected in prisons around the world and the reactivation of latent TB bacilli in the HIV-infected population has threatened our ability to control the spread of the disease [2]. In addition, the protective efficacy of the widely used TB vaccine *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been found to be highly variable [3]. Thus, the need for improved anti-TB drugs and vaccines is now more urgent than ever, as the lives of millions of people can be saved, and millions more can be protected

from ever contracting TB with effective vaccination against the disease.

Strains of *M. tuberculosis* that are attenuated for in vivo growth have been shown to provide protection against virulent strains [4]. Virulent strains, such as H37Rv and Erdman, grow more rapidly in mice than do attenuated strains, such as H37Ra and BCG [5]. Therefore, knowledge of genes that regulate the growth rate of *M. tuberculosis* may further our understanding of the relationship between bacterial expansion and the pathogenesis of TB. In the present study, we describe the role played by the gene MT2175 in growth and virulence in the mouse model of TB.

Some information is available on the biochemical properties of the protein encoded by MT2175. The gene Rv2115c is its homologue in *M. tuberculosis* H37Rv and shares 100% sequence identity. MT2175 and its homologue in *Mycobacterium leprae* (ML1316) show 81% sequence similarity to an AAA-ATPase-forming ring-shaped complex (ARC) of *Rhodococcus erythropolis* [6]. In silico domain analysis predicted an N-terminal coiled-coil motif and a C-terminal AAA-ATPase domain. The presence of an N-terminal coiled-coil domain, regarded as a hallmark of proteasomal AAA-ATPases, and the proximity of MT2175 to putative proteasomal genes sug-

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gest that this gene may be associated with proteasome function. Although the 20S proteasome is ubiquitously present in *Eukarya* and *Archea* species, its presence in bacteria is restricted to the phylum *Actinobacteria*, of which mycobacteria are members [7, 8]. Recent reports have suggested that the protein encoded by the homologue of MT2175 in *M. tuberculosis* H37Rv is a mycobacterial proteasome-associated ATPase that is involved in responses to nitrosative and oxidative stresses [9]. In addition, Darwin et al. studied the in vivo growth phenotype of this mutant in wild-type (WT) mice and inducible nitric oxide synthase knockout (iNOS^{-/-}) mice and found that iNOS^{-/-} mice were more susceptible than the WT mice to the mutant [10]. These observations suggest that the gene is involved in the response to the reactive nitric oxide stress imposed by the host.

During a genetic screening of *Himar1* transposon mutants of *M. tuberculosis*, we identified 3 independent mutants whose colonies were consistently smaller than WT colonies and that had insertions in the gene MT2175. To further assess this observation, and to delineate the role played by this gene in the physiological mechanisms of growth, we created a knockout strain of *M. tuberculosis* carrying a deletion of MT2175 (hereafter referred to as “ Δ MT2175”). Here, we describe a controlled characterization of the growth rate as well as virulence and transcriptional profiles of Δ MT2175 that demonstrate that it is impaired in in vitro and in vivo growth. In addition, we show that the mutant elicits a muted interferon (IFN)- γ response in the lungs of mice and can provide immune protection against virulent WT superinfection when used as a vaccine.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. CDC1551, a clinical isolate of *M. tuberculosis* [11], was used as the host strain and was grown in either Middlebrook 7H9 plates (supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% [vol/vol] oleic acid–albumin–dextrose–catalase) or Middlebrook 7H10 plates (Becton Dickinson) [12]. The strain Δ MT2175::*res-hygR-res* was prepared as described elsewhere [13], using pYUB854 into which 5' and 3' flanks of MT2175 were cloned. This strain was complemented by integrating a single copy of a full-length WT MT2175 carrying its own promoter, using pMH94 [14]. Growth assays were performed in drug-free medium under constant stirring, and colony forming units were counted after 4 weeks of incubation on 7H10 plates at 37°C.

Mouse infection. Three groups of 4–5-week-old female BALB/c mice were infected with aerosolized log-phase cultures (OD₆₀₀, 0.77–0.82) of WT *M. tuberculosis* CDC1551, Δ MT2175, and the complemented strain (28 mice/group). In vivo growth rates were determined by enumerating bacterial populations in the lungs of mice 1, 21, 49, and 112 days after infection. Twelve mice from each group were retained to assess the time required

to reach mortality (time to death). Severe combined immunodeficient Beige mice (Charles River) were used to study the role played by MT2175 in the absence of an adaptive immune response. Three groups of mice (13 mice/group) were infected with aerosolized cultures of WT *M. tuberculosis* CDC1551, Δ MT2175, and the complemented strain. Three mice from each group were killed to determine the infecting inoculum. Time-to-death data were collected from the remaining mice. Similarly, for vaccine experiments, 3 groups of BALB/c mice (18 mice/group) were immunized with a low-dose aerosol of Δ MT2175, BCG, or control (1 \times PBS) and were aerosol challenged on the 28th day with WT *M. tuberculosis* CDC1551. Three mice from each group were killed on the days after immunization and challenge to determine the dosages of the immunizing and challenge strains. The remaining mice were used to examine pathological correlates, to determine time to death, and to assess the protective efficacy of Δ MT2175.

IFN- γ assay. Homogenates of lungs from the mice infected with WT *M. tuberculosis* CDC1551, Δ MT2175, or the complemented strain were serially diluted in 1 \times PBS and filtered through 0.22-micron sterile syringe filters (Millipore), and 100 μ L of each sample filtrate was used to perform ELISAs in 96-well plates, with the antibody AN18 used for coating and the antibody R4-6A2 used for detection (Mabtech). This analysis was repeated 4 times, and purified mouse IFN- γ was included as an internal control.

Microarray and real-time reverse-transcription polymerase chain reaction (RT-PCR). Total bacterial RNAs from WT *M. tuberculosis* and Δ MT2175 were extracted from liquid cultures in log phase and were purified using RNeasy columns (Qiagen). The RNAs were used to prepare amino-allyl-conjugated cDNAs (Atlas Kit; Clontech), which were labeled with Cy3 and Cy5 monofluorescent dyes (Amersham Pharmacia). Equal quantities of Cy3- and Cy5-labeled cDNAs (prepared using RNAs from the WT or Δ MT2175 strain) were cohybridized (for 16 h at 55°C) onto microarray slides representing all open reading frames of *M. tuberculosis* (Operon Technologies); fluorescence from each probe was recorded using the GenePix 4000B system (Axon Instruments) and was analyzed using Microsoft Excel or the statistical platform R. This analysis was repeated 6 times with independently derived RNA samples. Quantification of mRNA transcripts of genes MT3159 and MT3389 was performed by RT followed by quantitative real-time PCR, using SYBR Green Supermix (Bio-Rad Laboratories). mRNA of sigma factor A was used as an internal control for amplification and normalization of real-time PCR data.

RESULTS

Reduction of growth rate caused by deletion of MT2175. We created a collection of 1425 *Himar1* transposon insertion mutants of *M. tuberculosis* CDC1551 and genotyped and screened

each mutant for colony size and morphological appearance [15]. A small number of these mutants produced unusually small colonies on enriched medium. Three of these independently created mutants had transposon insertions in the gene MT2175, but they were at 3 different positions (codons 55, 161, and 298). We also created a gene-replacement mutant that lacked >90% of the coding sequence of MT2175, to assess in detail the consequences of the absence of this gene. Additionally, we complemented this deletion knockout with a single copy of the full-length gene expressed from its native promoter (figure 1A). Although the colonies of Δ MT2175 were significantly smaller in size compared with those of the WT strain when grown under identical conditions, the full-size colony phenotype was completely restored in the complemented strain (figure 1B). We then determined the in vitro growth rates of the mutant strain, parental strain, and complemented strain by

enumerating the colony forming units and monitoring changes in the optical density of the liquid cultures of the strains (figure 1C). The average in vitro doubling times, calculated from 3 independent growth measurements in which the colony forming units were enumerated at regular time intervals, were 22 h for Δ MT2175 and 18 h for the WT strain. At 28 days after plating, the 18-h doubling time for the WT strain would be expected to produce colonies with an average volume of 49 mm³, whereas that for mutants would be 0.9 mm³ (colony volume = cell volume \times cell population = $[\pi \times \text{cell radius}^2 \times \text{cell length}] \times 2^{\text{division cycles}}$). Assuming that colonies are semi-hemispherical, this model predicts our mutant to give colonies ~4-fold smaller in diameter than those of the WT strain. As can be seen in figure 1B, our data correlate well with this prediction. We considered the possibility that the gene MT2175 may be involved in controlling cell size, and its deletion could

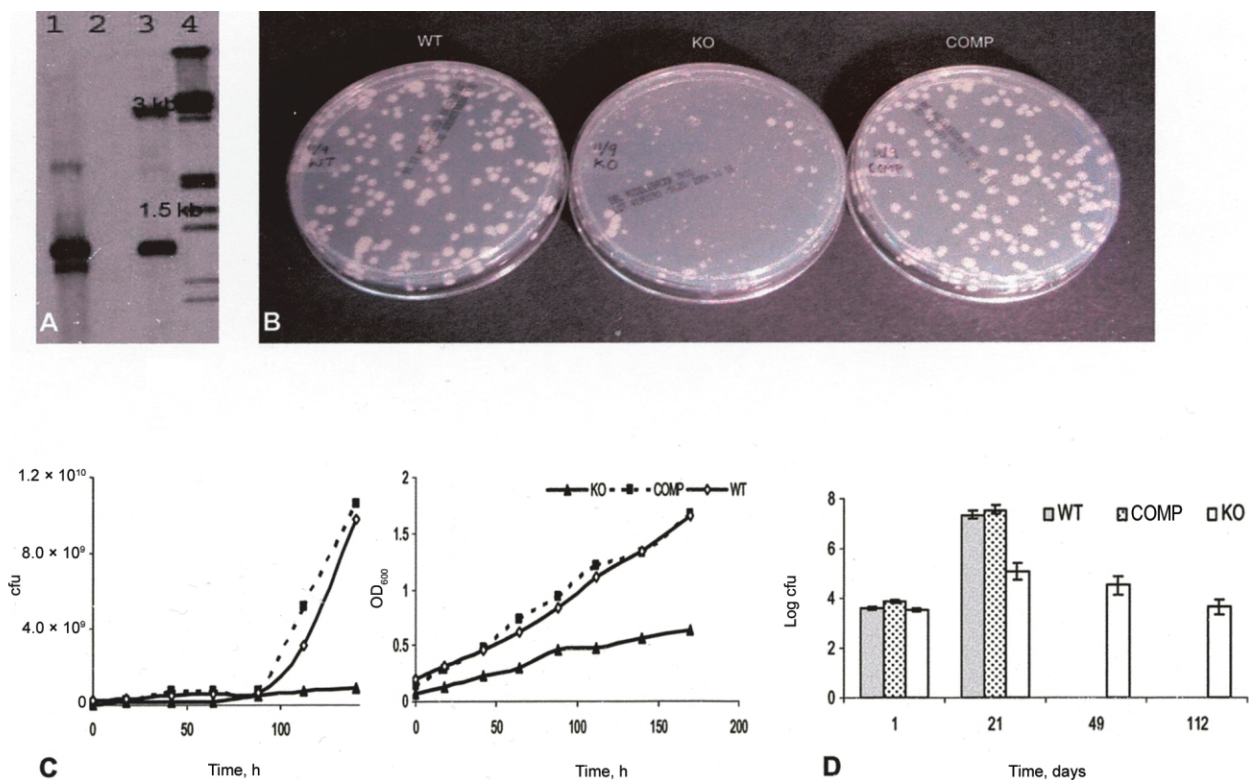


Figure 1. Strain characteristics. *A*, Southern blot analysis of genomic DNAs from wild-type (WT) *Mycobacterium tuberculosis* (lane 1), Δ MT2175 (lane 2), and the complemented (COMP) strain (lane 3). Lane 4 shows a marker. Genomic DNA was digested with *Sma*I and was probed with a labeled fragment of MT2175. The WT DNA shows the 2 expected fragments (1100 and 1240 bp), the Δ MT2175 DNA shows none, and the DNA from the complemented strain (which had a WT gene integrated at the *attB* site) shows the 2 predicted bands (1240 and 3048 bp). *B*, In vitro growth analysis 28 days after plating. Liquid cultures of the WT *M. tuberculosis*, Δ MT2175 (knockout [KO]), and the complemented strain were plated on Middlebrook 7H10 medium and photographed on the same day. The average colony size of Δ MT2175 was much smaller than that of the WT and the complemented strain. Each plate contained similar numbers of colony forming units. *C*, In vitro growth rates were analyzed by measuring the optical density (at 600_{nm}) of cultures of WT *M. tuberculosis*, Δ MT2175, and the complemented strain in Middlebrook 7H9 broth at regular time points (right). Aliquots of the culture were plated on Middlebrook 7H10 medium and incubated, and colony forming units were enumerated (left). *D*, Growth of WT *M. tuberculosis*, Δ MT2175, and the complemented strain in mouse lungs. Infected BALB/c mice were killed, and homogenates of lungs were plated on Middlebrook 7H10 medium, to obtain bacillary counts. Each plot represents the mean \pm SD number of colony forming units present in the lungs of 4 mice in each group.

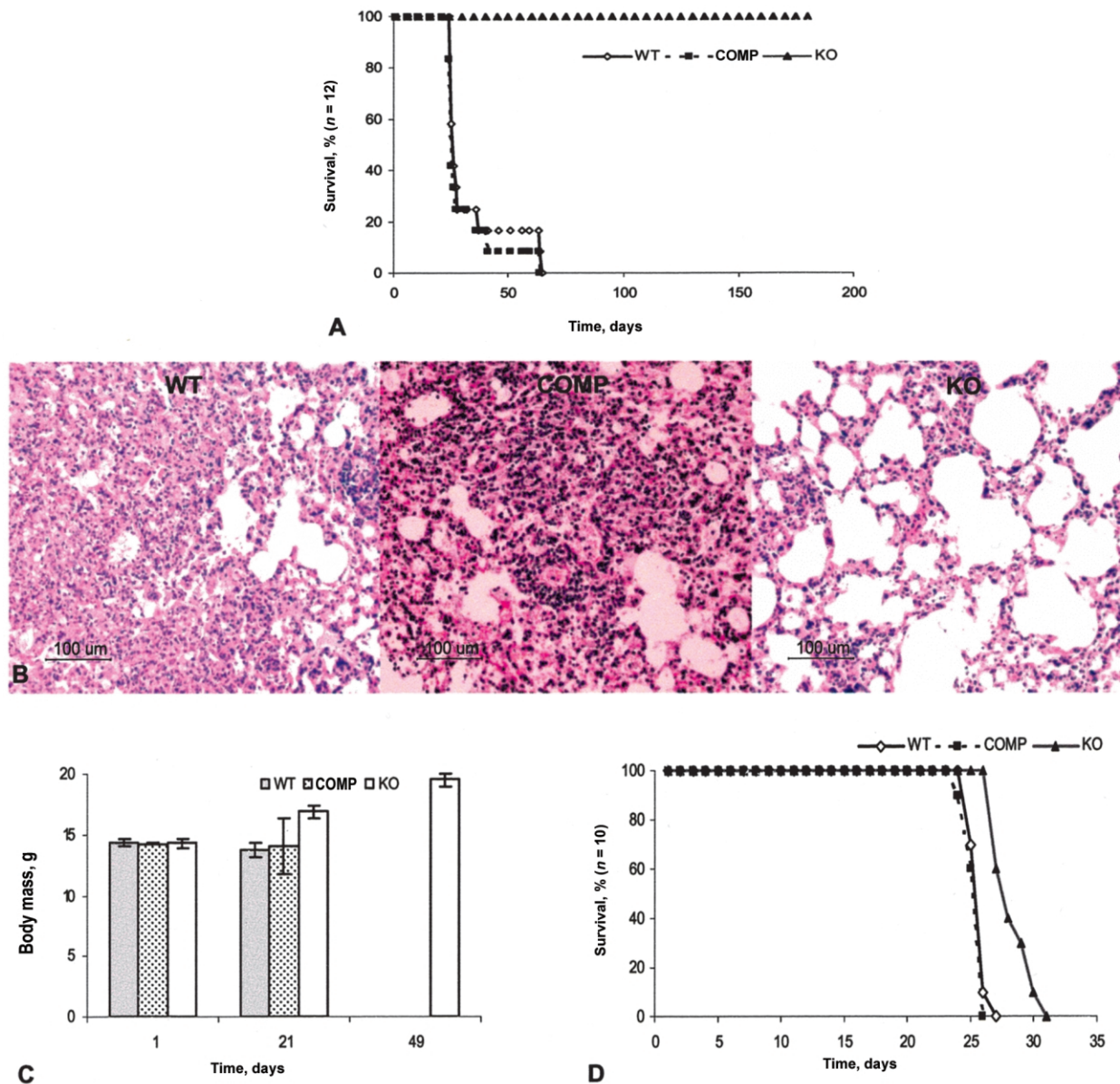


Figure 2. Time-to-death, histopathological, and body mass analysis. *A*, Time-to-death analysis of BALB/c mice. BALB/c mice were infected with liquid cultures of wild-type (WT) *Mycobacterium tuberculosis*, Δ MT2175 (knockout [KO]), or the complemented (COMP) strain, and time to death was recorded for each group. Twelve mice were used in each group. The median time to death for the mice infected with the WT or the complemented strain was 25 days. The mice infected with Δ MT2175 survived without any signs of disease (the experiment was terminated at 180 days after infection). *B*, Histopathological analysis of mouse lungs. BALB/c mice were killed 21 days after infection with WT *M. tuberculosis*, Δ MT2175, or the complemented strain. Sections of lower left lobes of the lungs were fixed, stained with hematoxylin-eosin, and observed at $\times 100$ magnification by light microscopy. *C*, Average body masses of BALB/c mice infected with WT *M. tuberculosis*, Δ MT2175, or the complemented strain. Body masses were recorded 1, 21, and 49 days after infection. Each bar represent the mean \pm SD mass of all mice alive in each group. *D*, Time-to-death analysis of severe combined immune deficient (SCID) mice. Three groups of SCID mice were infected with liquid cultures of WT *M. tuberculosis*, Δ MT2175, or the complemented strain, and time to death was recorded for each group. Ten mice were used per group.

result in smaller cells, leading to a smaller colony. We tested this hypothesis by scanning electron microscopy and found no difference in cell sizes; both strains measured $\sim 3.0 \mu\text{m}$ by $0.4 \mu\text{m}$ (data not shown).

Next, we assessed the in vivo growth rates of the 3 strains

after aerosol infection in immunocompetent BALB/c mice. Day 1 colony forming unit counts confirmed the implantation of ~ 3.6 log cfu of bacilli in the lungs of mice. Although Δ MT2175 was able to proliferate during the initial phase of pathogenesis, it increased by only ~ 1.5 log cfu, in contrast to the 3.5–4.0 log

cfu expansion observed in mice infected with the WT or the complemented strain (figure 1D). During the chronic phase of infection, from 21 to 112 days, Δ MT2175 demonstrated persistence with very gradually decreasing abundance, similar to other so-called persistence mutants [16]. These observations show that, in mice, the absence of the gene MT2175 leads to a growth-rate reduction and that there is an overall proliferation of Δ MT2175 during the initial phase, followed by slow clearance during the chronic phase of infection.

Δ MT2175 attenuated for lethality in immunocompetent mice. We then investigated the virulence of Δ MT2175 by assessing time to death and lung disease in BALB/c mice. Similar numbers of WT, Δ MT2175, and complemented strains were implanted at the time of infection, with a mean \pm SD of $\sim 3.6 \pm 0.1$ log cfu being recovered from the lungs 24 h after infection. As shown in figure 2A, the median time to death for the mice infected with either the WT or the complemented strain was 25 days, whereas the mice infected with Δ MT2175 survived without any signs of disease until 180 days, when the experiment was terminated. With respect to disease, the lungs of mice infected with the WT or the complemented strain showed intense inflammation, with many foci of granuloma-like cellular infiltrates replacing alveolar air spaces 21 days after infection (figure 2B). On the other hand, the lung parenchyma of mice infected with Δ MT2175 had reduced levels of immune infiltrates and ample alveolar air spaces. We also assessed the body weights of mice as a measure of the intensity of disease, as loss of body weight is a hallmark of disease progression. The mice infected with the WT or the complemented strain failed to gain body weight, although their lungs and spleens became progressively inflamed and gained mass (figure 2C). On the other hand, the mice infected with Δ MT2175 gained weight without any inflammatory gain in mass of their lungs and spleens. These observations led us to conclude that, although the loss of MT2175 produces a *M. tuberculosis* strain that is able to proliferate in immunocompetent mice, the mutant is severely compromised in its ability to produce lethality and the hallmark tissue damage associated with the murine model of TB. Thus, the Δ MT2175 mutant produces reduced immunopathological manifestations in mice, as has been described previously for such mutants as *M. tuberculosis* Δ sigH, Δ sigC, and Rel_{Mtb} [17–19].

Next, we studied the phenotype of Δ MT2175 in the absence of a host immune system. The median time to death for severe combined immune deficient (SCID) mice infected with either the WT or the complemented strain was 25 days, and that for the Δ MT2175-infected group was 27 days (figure 2D). Because similar numbers of colony forming units of the 3 strains were recovered 1 day after infection (mean \pm SD, $\sim 2.5 \pm 0.2$ log cfu), the 2-day time-to-death difference was not statistically significantly different. This pattern of full lethality in SCID mice

has been observed for other immunopathological mutants and is consistent with the contention that mutants of this class fail to elicit the same degree of immune-mediated tissue damage [17].

Lower levels of IFN- γ in mice infected with Δ MT2175. Because Δ MT2175 caused significantly reduced disease in the lungs, we investigated whether different host inflammatory responses were elicited after infection with the mutant versus the WT strain. BALB/c mice showed no differential response in IFN- γ production in the absence of MT2175 1 day after infection. The IFN- γ levels remained unchanged even at 21 days after infection in the mice infected with Δ MT2175, whereas the mice infected with the WT or the complemented strain had >4 -fold higher levels of IFN- γ (figure 3). These data correlated well with the observed histopathological manifestations: the lungs of the mice infected with the WT or the complemented strain were filled with large numbers of macrophages and lymphocytes, whereas the lungs of the mice infected with Δ MT2175 contained fewer infiltrates and a greater abundance of patent alveolar spaces (figure 2B). The levels of IFN- γ in the mice infected with the mutant strain were slightly higher at 49 and 112 days after infection but failed to reach the levels observed in the mice infected with the WT or the complemented strain. Thus, we conclude that the loss of the gene MT2175 leads to a reduced level of IFN- γ production and, hence, a milder Th1 immune response in mice.

Protection against virulent *M. tuberculosis* provided by immunization of mice with Δ MT2175. The decreased virulence of Δ MT2175, as evidenced by the diminished level of in vivo growth, reduced lung disease, and prolonged time to death, led us to assess its ability to provide immunological protection against WT *M. tuberculosis* infection. Three groups of mice were immunized with either BCG, Δ MT2175, or PBS (control), and the mean \pm SD colony forming unit counts in the lungs were 1660 ± 85 , 1460 ± 86 , and 0, respectively, 24 h after immunization. After 28 days, the mice were aerosol challenged with

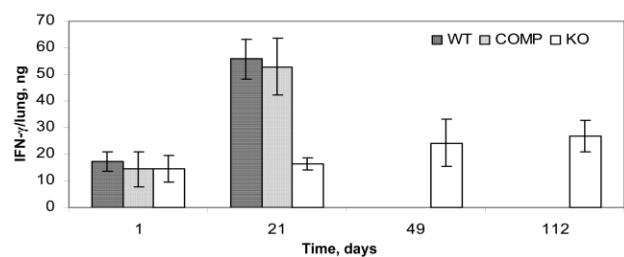


Figure 3. Levels of interferon (IFN)- γ produced in the lungs of mice infected with wild-type (WT) *Mycobacterium tuberculosis*, Δ MT2175 (knockout [KO]), or the complemented (COMP) strain, as determined by ELISA. Homogenates of lungs from mice killed 1, 21, 49, and 112 days after infection were used for the assay. Data are the mean \pm SD results of 4 independent assays.

WT *M. tuberculosis*, with a mean \pm SD of $\sim 4383 \pm 333$ cfu being recovered per mouse lung the following day. The median time to death for the control group was 40 days, whereas the groups immunized with either BCG or Δ MT2175 showed no signs of severe disease or fatality until 170 days after the challenge (figure 4); this difference was statistically significant ($P < .01$, Kaplan-Meier test). It is notable that this strain, which is derived from the virulent *M. tuberculosis* clinical isolate CDC1551, was able to protect against mortality due to TB and to extend the life of mice by ~ 120 days.

Differential up-regulation of genes *lat* (MT3389) and MT3159 in Δ MT2175. We studied changes in the transcriptome by comparing the levels of individual transcripts in WT and Δ MT2175 by use of whole genome microarrays. This experiment revealed 5 genes to be differentially up-regulated in Δ MT2175. Further analysis using quantitative real-time RT-PCR with gene-specific primers showed only 2 of the 5 genes to be consistently overexpressed in Δ MT2175. The mRNA levels of these genes—MT3159 (Rv3074) and *lat* (L-lysine epsilon aminotransferase; MT3389 [Rv3290c])—were higher by a mean \pm SD factor of 14.5 ± 8.1 and 12.9 ± 3.1 , compared with that in the WT strain. MT3159 (Rv3074) is annotated as a gene of unknown function. Its genomic locus shows that it is not a part of an operon, because the flanking genes are transcribed in opposite directions (<http://www.tigr.org>). A recent report suggests that this gene may be regulated by LexA, a protein involved in DNA excision and repair during SOS response [20]. A homologue of the gene *lat* is present in both *M. bovis* (Mb3318c) and *M. leprae* (ML0721) but is nonessential for the in vitro survival of *M. tuberculosis* [15, 21]. This gene has been implicated in metabolism, and a 42-fold increase in its expression under nutrient-starvation conditions has been reported [22]. The relation between these genes and MT2175 is not obvious from what is known and warrants further investigation.

DISCUSSION

Genes encoding putative 20S proteasome are present in species belonging to the phylum *Actinobacteria*, of which mycobacteria are members. Its function, however, is not essential for axenic growth of *Mycobacterium smegmatis* [23]. Recently, MT2175 (Rv2115c) was proposed to be one of the subunits of mycobacterial proteasome, on the basis of its sequence homology with ARC of *R. erythropolis*, close proximity to the gene *prcAB*, and the effect of proteasome inhibitors on the proteolytic activity of *M. tuberculosis* lysate on peptide substrates [9, 10]. Here, we report that the gene MT2175 is required for normal in vitro and in vivo growth and virulence of *M. tuberculosis*.

The pathogenesis of TB has been widely recognized as an interplay between the infecting bacterial mass and the host immune response. The recruitment of a robust supply of activated macrophages and lymphocytes in the lungs is believed to suppress bacterial multiplication and growth [24]. It is therefore reasonable to conjecture that reduced growth rate of the bacteria may permit the host enough time to generate an effective immune response and limit pathogenesis of the disease. We tested this hypothesis by infecting mice with WT *M. tuberculosis*, Δ MT2175, and a complemented strain. The reduced inflammation and disease in the lungs and spleens was exclusively observed in mice infected with Δ MT2175, which is characteristic of a class known as the “immunopathology mutants,” because they persist in the lungs and/or spleen but do not cause significant immune-induced disease. Deletion of genes such as *sigH*, *sigC*, and *Rel_{Mtb}* result in mutants that are capable of persisting without causing extensive disease and morbidity [17–19].

IFN- γ is an important immune effector molecule that is secreted primarily by T cells in response to *M. tuberculosis* infection, and it activates macrophages for intracellular killing. Reduced levels of IFN- γ have been reported in patients with active TB [25], and those with a defective IFN- γ receptor are

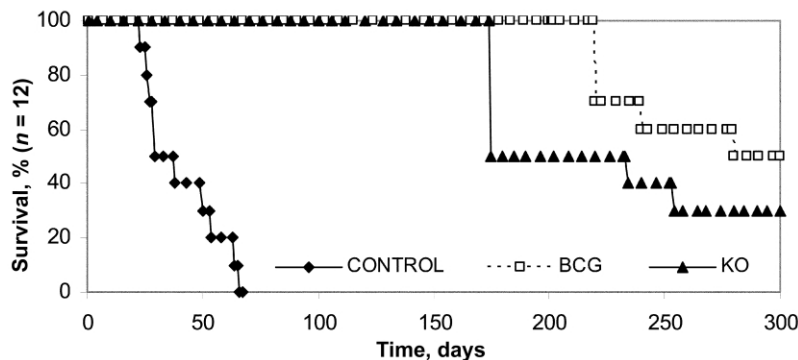


Figure 4. Efficacy of Δ MT2175 as a vaccine. Three groups of BALB/c mice ($n = 12$ /group) were immunized with either Δ MT2175 (knockout [KO]), *Mycobacterium bovis* bacille Calmette-Guérin (BCG), or 1 \times PBS (CONTROL). The mice were then infected with wild-type *Mycobacterium tuberculosis* after 28 days, and times to death were recorded. The median time to death in the control group was 40 days, whereas the mice immunized with either BCG or Δ MT2175 showed no signs of morbidity until ~ 170 days (the experiment was terminated at 300 days after challenge).

susceptible to infection with otherwise nonpathogenic mycobacteria [26]. Treatment with aerosolized IFN- γ has shown therapeutic value in patients infected with multidrug-resistant TB [27]. Mice lacking the gene for IFN- γ are susceptible to mycobacterial infection, because they develop granulomas and undergo extensive destruction of lung parenchyma, leading to premature death [28–30]. In addition, IFN- γ -deficient mice are unable to control bacterial growth and tissue necrosis but show delayed development of disease when being treated with exogenous IFN- γ [31]. In the present study, we observed that immunocompetent mice displayed a muted IFN- γ response when infected with Δ MT2175 and lived much longer than those infected with WT *M. tuberculosis*. Similar levels of IFN- γ were observed at the onset of infection in the lungs of mice infected with similar doses of the WT, Δ MT2175, or complemented strains. Yet, IFN- γ levels failed to increase only in the mice infected with Δ MT2175 during the acute phase of infection, despite an increase in colony forming unit counts (figures 1D and 3). It may be argued that rapid proliferation of either colony forming units, as exhibited by the WT or the complemented strain, or effector molecules that are not related to growth but that are affected by the loss of MT2175 is required to elicit higher levels of IFN- γ . Whether this effect is mediated directly by the loss of MT2175 or indirectly through other genes differentially affected by it, such as MT3159 and *lat*, remains to be elucidated.

The inability of Δ MT2175 to cause effective disease, morbidity, and mortality prompted us to examine whether it could provide immunity against the pathogenesis of TB caused by WT *M. tuberculosis*. In addition, the observation that Δ MT2175 can persist chronically would make it ideal for presenting antigens for a protracted period of time. Although the mouse model is believed to be inadequate to fully assess the immune properties of a prospective vaccine, Δ MT2175 and BCG did confer similar levels of protection against the pathogenesis of TB. The ability of Δ MT2175 to extend the life of mice by 120 days is noteworthy and, therefore, warrants further study in animals that form tuberculous granulomas and caseating cavities, to assess the relevance of protective efficacy of this strain in humans. Thus, Δ MT2175 shows interesting properties as a live attenuated vaccine for TB and could play a role in generating a safe and effective *M. tuberculosis*-derived vaccine [32].

We investigated the molecular mechanisms that may explain the growth attenuation and associated phenotypes of the mutant by examining its transcription profile and found 2 genes whose expression was highly induced in the mutant only. These genes, *lat* and MT3159, have not been associated with cell cycle regulation or growth. Although *lat* has been implicated in metabolism, it has been shown that this gene is not required for in vitro survival [15, 21]. The gene MT3159 encodes a protein of unknown function. In silico genomic and proteomic analysis

do not reveal any obvious association of these proteins with MT2175, thus warranting further study of this finding.

Here, we have presented data indicating that the loss of the gene MT2175 leads to a reduced in vitro and in vivo growth rate of *M. tuberculosis* and severely compromises its lethality in mice. The mutant lacking the gene failed to induce robust levels of IFN- γ and produced limited tissue damage in mice. The mutant persisted for an extended period of time in diminished numbers and, when used as a vaccine, provided immune protection against the pathogenesis of disease by virulent WT *M. tuberculosis*.

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