Genetic Requirements for the Survival of Tubercle Bacilli in Primates

Noton K. Dutta, Smriti Mehra, Peter J. Didier, Chad J. Roy, Lara A. Doyle, Xavier Alvarez, Marion Ratterree, Nicholas A. Be, Gyanu Lamichhane, Sanjay K. Jain, Michelle R. Lacey, Andrew A. Lackner, and Deepak Kaushal

Divisions of Bacteriology and Parasitology, Comparative Pathology, Microbiology, Veterinary Medicine, Tulane National Primate Research Center, Covington, and Departments of Mathematics and Biostatistics and Microbiology and Immunology, Tulane University, New Orleans, Louisiana; and Johns Hopkins Center for Tuberculosis Research, Baltimore, Maryland

Background. Tuberculosis (TB) leads to the death of 1.7 million people annually. The failure of the bacille Calmette-Guérin vaccine, synergy between AIDS and TB, and the emergence of drug resistance have worsened this situation. It is imperative to delineate the mechanisms employed by Mycobacterium tuberculosis to successfully infect and persist in mammalian lungs.

Methods. Nonhuman primates (NHPs) are arguably the best animal system to model critical aspects of human TB. We studied genes essential for growth and survival of M. tuberculosis in the lungs of NHPs experimentally exposed to aerosols of an M. tuberculosis transposon mutant library.

Results. Mutants in 108 M. tuberculosis genes (33.13% of all genes tested) were attenuated for in vivo growth. Comparable studies have reported the attenuation of only ∼6% of mutants in mice. The M. tuberculosis mutants attenuated for in vivo survival in primates were involved in the transport of various biomolecules, including lipid virulence factors; biosynthesis of cell-wall arabinan and peptidoglycan; DNA repair; sterol metabolism; and mammalian cell entry.

Conclusions. Our study highlights the various virulence mechanisms employed by M. tuberculosis to overcome the hostile environment encountered during infection of primates. Prophylactic approaches aimed against bacterial factors that respond to such in vivo stressors have the potential to prevent infection at an early stage, thus likely reducing the extent of transmission of M. tuberculosis.
M. tuberculosis mutants. A total of 326 M. tuberculosis CDC1551 Himarl mutants [6–8, 14] were used to infect NHPs in 3 pools. Each pool contained a positive (mutant in Rv1864c) and a negative (mutant in Rv1863c) control.

Macaque infection and veterinary procedures. The experiment was divided into 3 phases. In each phase, 4 adult male Indian rhesus macaques were infected with aerosols (dose, ∼10,000 colony-forming units [cfu]) of an identical pool of 100–120 mutants. A total of 326 unique mutants were used over the 3 phases. Aerosol exposures were performed on anesthetized animals in an unrestricted breathing configuration. Whole-body plethysmography was used to estimate respiratory minute volume prior to exposure [15, 16]. Two of the animals from each phase were euthanized 24 h after infection. These animals are hereafter referred to as the input group (CM77, DG82, BG22, DE81, CB10, and BD62). The mean age (± standard deviation [SD]) of the input group was 8.76 ± 1.31 years, and the mean weight (±SD) was 14.55 ± 3.01 kg. DNA isolated from pooled M. tuberculosis colonies obtained from the lungs of the animals in the input group was labeled with Cy5. The remaining 2 animals from each phase were followed up for the development of acute and fatal pulmonary TB (mean time to death [±SD], 28.7 ± 4.5 days). These animals (EN36, EK75, DV08, ER12, EI05, and DD77; mean age [±SD], 6.06 ± 1.24 years; mean weight [±SD], 8.19 ± 1.01 kg) are hereafter referred to as the output group. DNA prepared from the M. tuberculosis obtained from the lungs of the output group was labeled with Cy3. Equimolar quantities of input and output samples were used for DeADMAn. The M. tuberculosis mutants that fail to survive during pathogenesis of TB would be under represented in the output samples and directly reveal the identity of the genes required for the survival and multiplication of M. tuberculosis in primate lungs (Figure 1).

The animals were subjected to physical examination, chest radiographs (CXRs), and primagam interferon (IFN)-γ (Bio-cor) and serum C-reactive protein (CRP) assays at scheduled intervals. Primagam assay was performed as previously described, using 3 mL of whole lithium-heparin blood [17]. All procedures were approved by the relevant Institutional Animal Use/Care and Biosafety Committees.

Pathology. Necropsy and pathology procedures have been described elsewhere [17, 18]. Percentage of right lung involvement was determined by point counting using an overlaid grid (18.5-mm point spacing) on digital images of 3 random microscopic fields (original magnification, ×2.5) per slide. One sample from each of the 4 lobes of the right lung was used. Intersections representing normal lung included interstitium and air space, whereas lesions included intersections with inflammatory cells, hemorrhage, edema, and necrosis.

Microarray procedures. An entire lung from each animal was homogenized, decontaminated (Mycoprep; Becton Dickinson), and incubated on Middlebrook 7H11 agar at 37°C. Three weeks later, the colonies were scraped and pooled, and genomic DNA was processed [6–8]. Raw data were filtered, log-transformed, and normalized. Mutants with an input:output ratio >1.5-fold in a statistically significant manner (analysis of variance, P < .05) were considered to be attenuated.

Validation of microarray results. Real-time polymerase chain reaction (PCR) analysis in triplicate was used to confirm and quantify microarray results, as previously described [6–8].

Comparison of DeADMAn and TraSH in different models. We compared data from the murine in vivo [4] and murine-macrophage TraSH [5] experiments with the 326 mutants tested in NHPs. For the macrophage experiment, we used only the “unactivated” subset of data and considered genes to be attenuated when the in vivo/in vitro ratio was <0.67. For a head to head comparison of NHP and mouse DeADMAn, we infected 30 mice (5 mice per group) with 3 different pools of 107 identical mutants at 107 cfu and sacrificed at days 1 and 182 after infection. To assess the agreement across models, the phi correlation coefficient was computed for each pair, and χ² tests were performed. P values were adjusted for multiple testing using the Benjamini and Hochberg correction to control the false-discovery rate.

RESULTS

To identify M. tuberculosis genes required for survival and growth in vivo, we screened 326 unique, defined M. tuberculosis mutants in an NHP model of TB using the DeADMAn method [6–8].

Aerosol mediated infection of NHPs with M. tuberculosis and progression to acute TB. After aerosol infection, the input M. tuberculosis burden (expressed as mean cfu per g [±SD]) was obtained by harvesting NHP lungs 1 day after infection. These results showed comparable infection of all
NHPs in this group (588.25 ± 146.63 cfu/g). All animals in the
output group developed acute, fatal pulmonary TB. These an-
imals were euthanized within 4 weeks (mean time to sacrifice
[± SD], 28.7 ± 4.5 days) after infection because of pulmonary
disease. Their conditions were characterized by respiratory dis-
tress, fever, weight loss, and high levels of antigen-specific IFN-
γ in stimulated peripheral blood lymphocytes (Figure 2A–C).
Primagam values for each of the 6 animals in the output group

Figure 1. The Designer Arrays for Defined Mutant Analysis (DeADMAn) approach. Individual Mycobacterium tuberculosis (Mtb) mutants are used to form 3 distinct pools, each comprising an equal quantity of 100–120 mutants. Nonhuman primates (NHPs) are exposed to infectious aerosols of each pool and are either subjected to necropsy 24 h after infection (input group) or euthanized when they develop pulmonary tuberculosis (mean time to sacrifice [± standard deviation], 28.7 ± 4.5 days (output group). M. tuberculosis colony-forming units obtained from both groups of animals are then used for DeADMAn to identify M. tuberculosis genes required for survival in primate lungs.
Figure 2. Clinical measures of disease progression. A, Temporal changes of body temperature (°C) after exposure to infectious aerosol of *Mycobacterium tuberculosis* mutants. B, Percentage change in body weight. C, Whole-blood interferon (IFN)-γ release assay (Primagam, A450) at the time of necropsy. The results are shown as mean (± standard deviation) of triplicate experiment. The line perpendicular to the x axis and closer to y axis indicates the very likely presence of *M. tuberculosis* infection. A second line perpendicular to x axis and farther from the y axis indicates the acceptance range of mean absorbance of the primate IFN-γ positive control. D, Elevated serum C-reactive protein (CRP) level (mg/L). E, Confocal laser microscopy images show the lung sections of (i) animals belonging to the input pool (DE81) and (ii) animals belonging to the output pool (DV08) stained with *M. tuberculosis* (green) and macrophage (red) markers. 

were significantly higher than were those for the negative control group, which indicates likely *M. tuberculosis* infection, and were higher than the accepted level for the positive control group, thus confirming *M. tuberculosis* infection. In *M. tuberculosis*-infected NHPs, serum CRP values increase in a manner proportional to the extent of pulmonary pathology and inflammation [17, 19]. All animals from this output group exhibited levels of serum CRP that were significantly higher than preinfection levels (normal range, 0.1–1.0 mg/L) (Figure 2D), indicating severe systemic inflammation after infection with pools of *M. tuberculosis* mutants. The preinfection and week 1 and week 2 CXRs did not reveal any signs of tuberculous involvement, whereas the week 3 or week 4 CXRs revealed a pulmonary interstitial pattern with miliary nodules for all of the animals.

At necropsy, we compared the gross and microscopic pathology of the lung between the 2 groups. Although no lesions were observed in the lungs of the animals from the input group (because they were necropsied 24 h after *M. tuberculosis* exposure), granulomatous lesions were grossly apparent in all output animals, with thickened pleural surface and scattered ecchymotic hemorrhages (Figure 3A). The lesions ranged from solitary grayish-white nodules (1–3 mm in diameter) to coalescing miliary granulomas involving all lung lobes and bronchial lymph nodes (Figure 3A). Histological findings showed that the granulomas were characterized by dense aggregates of epitheliotid macrophages with a less dense peripheral lymphocytic infiltrate and a few multinucleated giant cells surrounding...
a central zone of necrotic debris and degenerating polymorphonuclear leukocytes (Figure 2B). Single and occasionally multiple extrathoracic granulomas were also seen in the liver, spleen, and kidney. The lungs of these animals exhibited high numbers of \( M. \) \( \text{tuberculosis} \)-associated granulomatous lesions, which resulted in high pulmonary pathology scores (Figure 3C).

Multiple-label confocal microscopy demonstrated the presence of \( M. \) \( \text{tuberculosis} \) within the granulomas (Figure 2E). At necropsy, we determined bacillary burden in the left lung from the output animals (Figure 3D). For 5 of 6 animals, the bacillary load was between \( 10^4 \) and \( 10^6 \) cfu per g of lung tissue, again confirming a comparable infection of all animals. All animals in the output group developed acute pulmonary TB characterized by uncontrolled bacillary replication and severe granulomatous pneumonia.

**Analysis of \( M. \) \( \text{tuberculosis} \) mutants attenuated for growth in macaque lungs.** Cy5- and Cy3-labeled input and output probes were mixed in equal amounts and co-hybridized onto

---

**Table 1. Mycobacterium tuberculosis Transposon (Tn) Mutants found to be Attenuated for Survival in the Nonhuman Primate (NHP) Aerosol Model**

This table is available in its entirety in the online version of the *Journal of Infectious Diseases.*
Table 2. Validation of Designer Arrays for Defined Mutant Analysis (DeADMAn) Microarray Results of Attenuated Mutants in Nonhuman Primate Lungs by Real-Time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutant attenuation, mean ratio (± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT2960</td>
<td>0.15 ± 0.06</td>
<td>.03</td>
</tr>
<tr>
<td>Rv0084</td>
<td>0.03 ± 0.02</td>
<td>.02</td>
</tr>
<tr>
<td>Rv0393</td>
<td>0.01 ± 0.007</td>
<td>.01</td>
</tr>
<tr>
<td>Rv0466</td>
<td>0.06 ± 0.03</td>
<td>.01</td>
</tr>
<tr>
<td>Rv0576</td>
<td>0.02 ± 0.01</td>
<td>.01</td>
</tr>
<tr>
<td>Rv0580c</td>
<td>0.0005 ± 0.0003</td>
<td>.02</td>
</tr>
<tr>
<td>Rv0595c</td>
<td>0.02 ± 0.01</td>
<td>.02</td>
</tr>
<tr>
<td>Rv0954</td>
<td>0.001 ± 0.0008</td>
<td>.01</td>
</tr>
<tr>
<td>Rv1176c</td>
<td>0.02 ± 0.01</td>
<td>.02</td>
</tr>
<tr>
<td>Rv1185c</td>
<td>0.18 ± 0.09</td>
<td>.03</td>
</tr>
<tr>
<td>Rv1371</td>
<td>0.03 ± 0.01</td>
<td>.01</td>
</tr>
<tr>
<td>Rv1704c</td>
<td>0.05 ± 0.02</td>
<td>.01</td>
</tr>
<tr>
<td>Rv1863c</td>
<td>1.30 ± 0.25</td>
<td>.02</td>
</tr>
<tr>
<td>Rv1864c</td>
<td>0.28 ± 0.11</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Rv1978</td>
<td>0.02 ± 0.01</td>
<td>.02</td>
</tr>
<tr>
<td>Rv2072c</td>
<td>0.19 ± 0.21</td>
<td>.02</td>
</tr>
<tr>
<td>Rv2387</td>
<td>0.004 ± 0.002</td>
<td>.01</td>
</tr>
<tr>
<td>Rv2459</td>
<td>0.22 ± 0.12</td>
<td>.04</td>
</tr>
<tr>
<td>Rv2796c</td>
<td>0.12 ± 0.06</td>
<td>.01</td>
</tr>
<tr>
<td>Rv2936</td>
<td>0.07 ± 0.06</td>
<td>.05</td>
</tr>
<tr>
<td>Rv3197</td>
<td>0.04 ± 0.02</td>
<td>.02</td>
</tr>
<tr>
<td>Rv3297</td>
<td>0.0003 ± 0.0002</td>
<td>.02</td>
</tr>
<tr>
<td>Rv3327</td>
<td>0.05 ± 0.02</td>
<td>.02</td>
</tr>
<tr>
<td>Rv3741c</td>
<td>0.005 ± 0.002</td>
<td>.02</td>
</tr>
<tr>
<td>Rv3787c</td>
<td>0.01 ± 0.007</td>
<td>.02</td>
</tr>
<tr>
<td>Rv3871</td>
<td>0.01 ± 0.009</td>
<td>.02</td>
</tr>
</tbody>
</table>

NOTE. Survival of mutants in nonhuman primate lungs. Attenuation for each mutant is expressed as the ratio of mutants present in the input pool to mutants present in the output pool; a ratio >1 was negative and <1 was positive (P<.05).

* Negative control.
* Positive controls were present in all pools and yielded the same results.

DeADMAn microarrays. With use of quantitative PCR, we identified mutants with invariant levels between the input and output samples in all 3 phases of the experiment. The nucleotide abundance of a mutant in Rv1863c gene (JHU1863c-275), which had been used as a negative control (a mutant that is not attenuated) during prior studies [6], was relatively unchanged after in vivo growth in primate lungs (mean output: input ratio [± SD], 1.32 ± 0.23). This mutant was used for rank-invariant normalization of microarray data. A clustered heat-map shows attenuated mutants with diminished output signal, colored in red (Figure 4). Of the 326 total mutants that we tested in 3 phases, 108 (33.13%) were attenuated for growth (Table 1). This number is significantly higher than that found in comparable mouse studies (6%) [6].

Of the 7 mutants in genes involved in mammalian cell entry (mce) mutants tested in our screen, 5 (mce1E, yrbE3B, mce3A, mce4F, mce4E) were attenuated for growth in primate lungs. In addition, mutants in genes involved in lipid metabolism (fadD30, lipU, pks4, fadD21, Rv1371, mbfJ, and lipQ); biosynthesis of lipoarabinomannan (embc), cobalamin (cobI), molybdenum (moaC1), and thiosulfate (cysA3); excision repair (uvrB, fpg, and nei); transport (mntH, Rv1200, cyaA, Rv2041c, Rv2459, yajC, Rv2690c, amt, drrA, and Rv3197); transcription (regX3, Rv0576, and phoY1); stress or immune response (Rv0954, Rv1057, Rv1234, Rv1264, cadI, PE_PGRS47, PPE53, and Rv3871); and metal ion sequestering (Rv2850c) were also attenuated. Twenty-four mutants attenuated in the DeADMAn screen were randomly selected for validation with quantitative real-time DNA PCR, along with the two mutants that were used as the positive and the negative control. We were able to validate the attenuation of all of these mutants via PCR (Table 2).

M. tuberculosis mutants not attenuated for survival and growth in primate lungs. Mutants in several genes involved in the biosynthesis of biotin (bioF2), purines (glyA2), trehalose (treS), cysteine (cysB), polyamines (adi), phospholipids (gspa), triacylglycerol (tgS1) and guanosine 5’-monophosphate (guaB3), intermediary metabolism (Rv0458, Rv0480c, Rv0696, fucA, mapA, Rv0895, zwf1, amiB1, amiB2, glgP, Rv1393c, epiA, Rv1526c, Rv1869c, amiA2, Rv3049c, Rv3085, Rv3175, and Rv3224), isoniazid resistance (inaA), heat shock (htpX), regulation (kdpE, sirR, moxR3, and sigF), starvation, cell division, DNA degradation, and transport, and some toxin-antitoxin genes (Rv0550c, Rv0659c, and Rv0662c) were also not attenuated for survival in NHP lungs. Mutants in several genes with predicted roles in virulence and pathogenesis were not attenuated in NHP lungs. These included mutants in genes encoding ESAT-6 homologs (essD and essF), a virulence factor involved in the transport of adhesion component (Rv0986) [20], hemolysin (Rv0986), multidrug ABC transporters (Rv1273, Rv1348, and Rv2333c), a nitrate reductase involved in persistence (narX) [21], a virulence factor (ephB), polyketide synthase (pks12) [22], a protein involved in the intracellular removal of iron from iron-mycobactin complex (viuB), and response to hypoxia (dosS) [23]. Seventeen of the 20 mutants in genes belonging to the PE/PPE-PGRS families were not attenuated. This is in contrast to prior studies in which 100% of tested mutants from this functional category were found to be not attenuated in mice [6] and guinea pigs [7].

Cross-species comparison of macaque DeADMAn data. The NHP data was compared with the mouse DeADMAn data (Figure 5A and 5B), as well as with historical data obtained from TraSH in murine macrophages (Figure 5A and 5C) [5] and mice (Figure 5C), respectively. To compare identical DeADMAn mutants across different species, we chose a subset of the 326 mutants that had been tested in NHPs and infected...
Figure 5. Venn diagrams show the degree of association between the attenuated mutants of Mycobacterium tuberculosis genes in different models. A, Of 108 tested mutants, 9 and 5 M. tuberculosis transposon (Tn) mutants, attenuated for survival in the macrophage transposon site hybridization (TraSH) and mouse Designer Arrays for Defined Mutant Analysis (DeADMAn) models, respectively, are common with the nonhuman primate (NHP) DeADMAn model. B, List of M. tuberculosis Tn mutants, attenuated for survival in both the monkey and mouse aerosol DeADMAn models. C, Of 326 tested mutants, 10 and 19 M. tuberculosis Tn mutants, attenuated for survival in the mouse and macrophage TraSH models, respectively, were also attenuated in NHPs. Significant pair-wise association between different experiments and models was tested based on a χ² test and phi coefficient.

DISCUSSION

Two critical aspects of human TB—latent disease and a wide spectrum of pathological features—are accurately captured by NHPs infected with M. tuberculosis [9–13]. Both Macaca mulatta [13, 18, 19] and Macaca fascicularis [9–12, 17] have been extensively used to model TB. It is believed that the latter are more resistant to M. tuberculosis infection, compared with the former. This assertion is based on observations that M. fascicularis can be better protected against a challenge with virulent M. tuberculosis by bacille Calmette-Guérin vaccination than M. mulatta, although this has recently been refuted [19]. In accordance with this understanding, M. fascicularis have primarily been used to model latency [10–13], whereas M. mulatta have been used for pathogenesis and vaccine-testing studies [13, 18, 19].
In macaques infected with \( M. \) \( \text{tuberculosis} \), granulomas may be caseous, cavitary, or cellular [24]. Hypoxic granulomas are not seen in mice [25]. The study of \( M. \) \( \text{tuberculosis} \) immunity and pathology in host species with predominantly nonhypoxic lesions can be fundamentally misleading [26]. Because of the differences in pathology of TB in mice and primates and the close genomic, physiological, and immunological similarities among primates, NHPs are an excellent model of TB. NHPs serve as internal controls of the consistency of our data [17, 19]. For these compelling reasons, an analysis of the pathogen's genetic requirements to survive and multiply in host species with predominantly nonhypoxic tissues is best performed in NHPs.

We infected NHPs with a very high dose of \( M. \) \( \text{tuberculosis} \), \( \sim 10^{10} \) cfu. These results ascribe value to studying \( M. \) \( \text{tuberculosis} \) virulence in the NHP model, in addition to its use for preclinical testing. A total of 38.58% of all mutants in genes that belonged to the “cell-wall associated” category were attenuated for survival (Table 3). The 2 genotype-identical mutants, HG0305 and HG0309 (JHU0157A-17) (Table 2), were both attenuated (4.24-fold and 4.92-fold, respectively). The mutants JHU2387–408 and JHU2387–151, affecting the same gene but at different positions (at nucleotides 408 and 151, respectively), were both attenuated (4.24-fold and 4.92-fold, respectively). The mutants JHU2960a–91 was a part of 2 different pools (Table 1) and was attenuated in both (7.76-fold and 7.13-fold, respectively). The 2 genotype-identical mutants, HG0305 and HG0309 (JHU0157A-17) (Table 2), were both attenuated (4.24-fold and 4.92-fold, respectively). The mutants JHU2387–408 and JHU2387–151, affecting the same gene but at different positions (at nucleotides 408 and 151, respectively), were both attenuated (4.19-fold and 10.28-fold, respectively). These mutants served as internal controls of the consistency of our data.

The attenuation of a much larger number of \( M. \) \( \text{tuberculosis} \) mutants (33.13%) was observed in NHP lungs, relative to comparable mice and guinea pig studies. Conceivably, the NHP immune system generates a higher degree of stress on the pathogen than does the the mouse immune system, thereby rendering a larger number of \( M. \) \( \text{tuberculosis} \) mutants attenuated. These results ascribe value to studying \( M. \) \( \text{tuberculosis} \) virulence in the NHP model, in addition to its use for preclinical testing. A total of 38.58% of all mutants in genes that belonged to the functional category “cell-wall associated” were attenuated for growth in the acute model of primate TB; 35.40% and 26.47% of the 326 mutants, the dose was determined on the basis of the existing DeADMAn model of mouse infection (\( 10^9 \)–\( 10^{10} \) cfu). Mutants were considered to be attenuated if a 50% reduction in normalized signal was observed in each of the 4 replicate spots on a microarray, as well as for each biological replicate in a statistically significant manner (\( P \leq .05 \), by analysis of variance).

The 2 genotype-identical mutants, HG0305 and HG0309 (JHU0157A-17) (Table 2), were both attenuated (4.24-fold and 4.92-fold, respectively). The mutants JHU2387–408 and JHU2387–151, affecting the same gene but at different positions (at nucleotides 408 and 151, respectively), were both attenuated (4.19-fold and 10.28-fold, respectively). These mutants served as internal controls of the consistency of our data.

Table 3. Functional Classification of the Genes Represented by the Mycobacterium tuberculosis Transposon (Tn) Mutants

<table>
<thead>
<tr>
<th>Functional category</th>
<th>No. tested</th>
<th>( M. ) ( \text{tuberculosis} ) Tn mutants</th>
<th>Attenuated for survival</th>
<th>Attenuation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Virulence)</td>
<td>17</td>
<td>Rv0240, Rv0595c, yrbE38, mce1A, mce4F, lprN</td>
<td></td>
<td>35.30</td>
</tr>
<tr>
<td>1 (Lipid metabolism)</td>
<td>10</td>
<td>faeO030, pkx4, fae21, lipK</td>
<td></td>
<td>40.00</td>
</tr>
<tr>
<td>2 (Information pathways)</td>
<td>10</td>
<td>( \text{regulon} )</td>
<td></td>
<td>30.00</td>
</tr>
<tr>
<td>3 (Cell wall related)</td>
<td>70</td>
<td>mce1E, lprQ, Rv0381c, lprP, mntH, Rv0954, Rv1200, Rv1234, Rv1371, Rv1433, cycA, lppT, Rv2041c, lppl, Rv2077c, Rv2459, yacC, Rv2597, Rv2686c, Rv2690c, lppN, dacB1, amt, dnrA, Rv3197, embBc, Rv3871</td>
<td></td>
<td>38.58</td>
</tr>
<tr>
<td>5 (Insertion sequences/phages)</td>
<td>7</td>
<td>Rv0336, Rv0393, Rv3327, Rv3430c</td>
<td></td>
<td>57.15</td>
</tr>
<tr>
<td>6 (PE/PPE)</td>
<td>20</td>
<td>Rv0872c, Rv2741, Rv3159c</td>
<td></td>
<td>15.00</td>
</tr>
<tr>
<td>7 (Intermediary metabolism)</td>
<td>68</td>
<td>Rv0069, hycD, Rv0213c, Rv0654, fabG, arcA, lpaU, Rv1264, tal, colB, lpoQ, hfx, Rv2890c, moaC1, cysA3, Rv3727, Rv3741c, lipE</td>
<td></td>
<td>26.47</td>
</tr>
<tr>
<td>9 (Regulatory proteins)</td>
<td>11</td>
<td>regK3, Rv0576, mce4E</td>
<td></td>
<td>27.28</td>
</tr>
<tr>
<td>10 (Conserved hypothetical)</td>
<td>113</td>
<td>cadJ, MT1029.3, MT1622.1, MT1650.1, MT2316, MT2375, MT2960, MT2960, MT3037, MT3536, Rv0079, Rv0157A, Rv0157A, Rv0162, Rv0250c, Rv0466, Rv0502, Rv0580c, Rv0910, Rv0948c, Rv1176c, Rv1489, Rv1804c, Rv1810, Rv1864c, Rv1879, Rv1894c, Rv1978, Rv2091c, Rv2309A, Rv2387, Rv2387, Rv2478c, Rv2694c, Rv2819c, Rv2820c, Rv2879c, Rv3094c, Rv3486, Rv3683, Rv3787c</td>
<td></td>
<td>35.40</td>
</tr>
<tr>
<td>Total</td>
<td>326</td>
<td></td>
<td></td>
<td>33.13</td>
</tr>
</tbody>
</table>

**NOTE.** PE, proline-glutamic acid; PPE, proline-proline-glutamic acid. Functional categorization of \( M. \) \( \text{tuberculosis} \) genes is based on the information available in Tuberculist database [27].
Rv0178, and its mutant is hypervirulent in mice, with a diminished ability to induce a Th1-type immune response [28]. The M. tuberculosis genome contains 3 homologous copies of mce1 (mce2, mce3, and mce4), which play important roles in M. tuberculosis virulence that are distinct from the role played by mce1 [29]. The expression of mce1 has recently been linked to sigH, a key M. tuberculosis stress response factor [30, 31]. The mce4 locus regulates cholesterol transport [32, 33]. Such mutants would be essential for survival in vivo, especially in primate tissues, where the pathogen experiences altered availability of nutrients and must adapt to glucose-deficient and fatty acid–abundant conditions. A mutant in a steroid dehydrogenase coding gene (Rv0687) was also attenuated. It has been suggested that M. tuberculosis can use sterols as a carbon source [34, 35]. It is possible that intracellular growth in primate lungs requires M. tuberculosis to adapt to a sterol utilization program, thus making a mutation in this pathway lethal. Adaptation to glucose-deficient conditions requires an active \( \beta \)-oxidation cycle, which results in high levels of propionyl-coA. This metabolite is a key precursor in several lipid biosynthetic pathways but is toxic in high amounts. It is detoxified via methylmalonyl-coA mutase, which requires cobalamin as a cofactor [36]. Based on the observed attenuation in the cobL mutant, it appears that cobalamin is essential for the in vivo survival of M. tuberculosis in primate lungs. These findings show that cholesterol biosynthesis and transport may play a crucial role in the adaptation of the pathogen within primate host tissues. Furthermore, they suggest the notion that primate lungs present a glucose-deficient, fatty acid–rich nutrient environment, and adaptation to such conditions is critical for the pathogen to survive.

A mutant in the Rv3871 gene was attenuated for growth in both primate (the present study) and murine lungs [6]. In fact, the Rv3871 mutant was the only one among those we tested that was attenuated in mouse and NHP DeADMan experiments, as well as in mouse [4] and murine macrophage [5] TraSH experiments, thus pointing to the central role for the Esx secretion pathway [37] involving this gene in virulence and pathogenesis of host tissues by M. tuberculosis. Rv3871 is involved in the secretion of ESAT-6 and CFP-10 protein antigens. Macrophages infected with the \( \Delta \)-Rv3871 mutant are highly activated and generate higher immune responses relative to M. tuberculosis, indicating that Rv3871-mediated secretion of protein antigens is required for the modulation of the host immune response by M. tuberculosis [37].

In conclusion, our experiments represent an initial attempt to study the effects of genetic mutations in M. tuberculosis with use of primate hosts. We show the feasibility of performing such studies using NHPs infected via the aerosol route. Our results show the crucial role of the mce and esx regulons and sterol biosynthesis and transport in facilitating the survival and multiplication of M. tuberculosis in primate host tissues. M. tuberculosis is an intracellular pathogen that is highly adapted for survival and growth in primate lungs. A study of its genetic requirements in NHPs therefore allows us to gain a better understanding of the bacterial factors that are likely to play a key role in facilitating virulence and pathogenesis. This work will provide researchers with a compendium of M. tuberculosis genes, regulons, and pathways that can serve as targets for future drug or vaccine development.

References

18. Gormus BJ, Blanchard JL, Alvarez XH, Didier PJ. Evidence for a rhesus


23. Kumar A, Toledo JC, Patel RP, Lancaster JR, Steyn AJ. Mycobacterium tuberculosis DosS is a redox sensor and DosT is a hypoxia sensor. Proc Natl Acad Sci USA 2007; 104:11568–11573.


