MECHANISMS OF PATHOGENESIS

The LuxR family regulator Rv0195 modulates Mycobacterium tuberculosis dormancy and virulence

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Summary

Tuberculosis is a leading global killer that has not been effectively controlled to date. The ability of the causative agent, Mycobacterium tuberculosis, to become dormant is one of the major reasons for extended chemotherapeutic regimens and wide epidemicity. The underlying mechanisms of M. tuberculosis dormancy are not fully understood. In the present work, a LuxR family transcription factor gene, Rv0195, was deleted in the virulent M. tuberculosis strain H37Rv. Rv0195 deletion did not affect bacterial growth and long-term survival under aerobicosis but decreased cell survival and the ability to rapidly recover from dormancy in an in vitro anaerobiosis model. The deletion also reduced intracellular survivability under hypoxic and reductive stress triggered by vitamin C. Microarray hybridization analysis showed that Rv0195 affected the expression of more than 180 genes under anaerobiosis, and these genes did not overlap with the known anaerobiosis-up-regulated DosR regulon genes. Furthermore, the Rv0195 deletion diminished bacterial virulence in human macrophage-like cells and resulted in reduced bacterial survival and pathogenicity in a C57BL/6 mouse infection model. These findings offer a novel insight into the mechanisms by which M. tuberculosis adapts to and recovers from dormancy and demonstrate that the dormancy regulator Rv0195 contributes to bacterial virulence.

\begin{abstract}
Tuberculosis is a leading global killer that has not been effectively controlled to date. The ability of the causative agent, Mycobacterium tuberculosis, to become dormant is one of the major reasons for extended chemotherapeutic regimens and wide epidemicity. The underlying mechanisms of M. tuberculosis dormancy are not fully understood. In the present work, a LuxR family transcription factor gene, Rv0195, was deleted in the virulent M. tuberculosis strain H37Rv. Rv0195 deletion did not affect bacterial growth and long-term survival under aerobicosis but decreased cell survival and the ability to rapidly recover from dormancy in an in vitro anaerobiosis model. The deletion also reduced intracellular survivability under hypoxic and reductive stress triggered by vitamin C. Microarray hybridization analysis showed that Rv0195 affected the expression of more than 180 genes under anaerobiosis, and these genes did not overlap with the known anaerobiosis-up-regulated DosR regulon genes. Furthermore, the Rv0195 deletion diminished bacterial virulence in human macrophage-like cells and resulted in reduced bacterial survival and pathogenicity in a C57BL/6 mouse infection model. These findings offer a novel insight into the mechanisms by which M. tuberculosis adapts to and recovers from dormancy and demonstrate that the dormancy regulator Rv0195 contributes to bacterial virulence.
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1. Introduction

Mycobacterium tuberculosis is notorious as the causative agent of tuberculosis, which results in approximately two million deaths every year around the world despite the availability of antituberculosis drugs. It is estimated that one-third of the world's population is latently infected with M. tuberculosis\textsuperscript{1}; this population represents a large reservoir for active tuberculosis, as about 10% of latent infections ultimately develop into active tuberculosis.\textsuperscript{2} It is thought that a certain proportion of infected individuals who remain asymptomatic harbor dormant bacilli\textsuperscript{2} that are tolerant to antituberculosis drugs such as isoniazid (INH) and rifampin.\textsuperscript{3,4} Dormancy of M. tuberculosis is thus a major cause for extended chemotherapeutic regimens and the wide epidemicity of tuberculosis.\textsuperscript{5}

The current explanation for M. tuberculosis dormancy involves the DosR (originally designated DevR\textsuperscript{20}) regulon, which consists of about 50 genes that are regulated by the two-component system DosR-DosS/T.\textsuperscript{5–8} Expression of the DosR regulon genes is readily induced after a variety of conditions are sensed through DosS/T, including exposure to hypoxia,\textsuperscript{5,7–10} NO\textsuperscript{11} and CO,\textsuperscript{11} and infections in macrophages,\textsuperscript{12} mice\textsuperscript{13} and guinea pigs,\textsuperscript{14} which possibly triggers M. tuberculosis dormancy. However, the expression patterns of the DosR regulon are divergent for the H37Rv, H37Ra\textsuperscript{15} and the W-Beijing lineage strains.\textsuperscript{16} Disruption of dosR does not result in the rapid death of M. tuberculosis during hypoxia\textsuperscript{7,8} and anaerobiosis.\textsuperscript{10} These observations suggest that the DosR regulon is not solely responsible for bacterial dormancy and survival.

Here, we report that a LuxR family regulator is involved in M. tuberculosis dormancy. LuxR transcriptional factors are widespread among bacteria and have a helix–turn–helix DNA binding domain at their C-terminal ends.\textsuperscript{17} In Gram-negative proteobacteria, LuxR proteins usually modulate population density by sensing diffusible N-acyl homoserine lactone (AHL) molecules.\textsuperscript{18,19} Typically, the LuxI and LuxR protein families are most commonly associated with AHL synthesis and the AHL signal response, respectively.\textsuperscript{20} In non-AHL-producing bacteria (almost 20% of microbes containing LuxR proteins), LuxR proteins can detect and respond to other molecular signals or AHLs produced by other species.\textsuperscript{21} LuxR proteins are divergent in terms of their primary
sequences and can exert diverse functions on various bacteria. The genome of *M. tuberculosis* H37Rv harbors 7 luxR genes, including *Rv0386*, *Rv0195*, *Rv0491*, *Rv0890c*, *Rv0894*, *Rv2488c*, and *Rv3133c* (dosR). Loss of *Rv0386*, an adenylate cyclase, decreases immunopathology in animal tissues and bacterial survival.23 The physiological roles of 5 other luxR genes remain unclear. We generated a deletion mutant of *Rv0195* in H37Rv and showed that *Rv0195* is involved in bacterial hypoxic/anaerobic dormancy. We further demonstrated that *Rv0195* mediates bacterial virulence in human macrophage-like cells and murine tissues.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*M. tuberculosis* H37Rv (ATCC27294, laboratory stock) was grown in 7H9 medium (Difco Laboratories, USA) containing 0.05% Tween 80 supplemented with 10% oleic–albumin–dextrose complex (OADC). Viabilities of *M. tuberculosis* cultures were measured in colony-forming units (CFUs) by plating bacterial dilutions onto 7H10-OADC agar plates. *Escherichia coli* DH5α was used as a host strain for cloning and plasmid propagation and was grown on Luria–Bertani (LB) medium.

2.2. Generation of the mutant and complemented strains

H37Rv was used as the parental strain to generate the mutant. Hygromycin (80 μg/ml), kanamycin (20 μg/ml), gentamicin (20 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 50 μg/ml) and sucrose (2% wt/vol) were used where appropriate. The deletion delivery suicide vector for the open reading frame (ORF) of *Rv0195* was constructed using the p1NIL and pGOAL19 vectors as previously described.24 Briefly, 1-kb fragments upstream and downstream of *Rv0195* were amplified by polymerase chain reaction (PCR) with the primer pairs 5F-KS/5F-A and 3F-S/3F-KA (Table 1), respectively. The two products were ligated to form an insertion fragment without the *Rv0195* ORF by PCR with the primer pair 5F-KS/3F-KA. After digestion by HindIII, the resulting fragment was inserted into the p1NIL vector and screened by PCR, and a lack of mutations was confirmed by DNA sequencing. The resulting plasmid was then integrated with the *pac* cassette (hyg-lacZ-sacB) from pGOAL19 to generate the suicide delivery vector pNG195. The pNG195 plasmid was pre-treated with alkali and transformed into *M. tuberculosis* H37Rv (ATCC27294, laboratory stock) and *M. tuberculosis* strain for cloning and plasmid propagation and was grown on 7H10-OADC agar plates. *Escherichia coli* DH5α was used as a host strain for cloning and plasmid propagation and was grown on Luria–Bertani (LB) medium.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used in this study.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Description</td>
</tr>
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<td>Primers for suicide knockout plasmid construction</td>
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<tr>
<td>5F-KS</td>
<td>CCCaactcGGTACGCGTGCGTAGC</td>
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<tr>
<td>5F-A</td>
<td>TTGCACCCGGTCCGGACG</td>
</tr>
<tr>
<td>3F-KA</td>
<td>CCCaagcttCGGAGGTTCAGCCG</td>
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<td>Primers for validation of candidate <em>Rv0195</em> mutants</td>
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<tr>
<td>v5</td>
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<tr>
<td>vA</td>
<td>GCCTCCTGTACAGGATTITCTTG</td>
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<td>Primers for gene-complemented plasmid construction</td>
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<tr>
<td>Com-S</td>
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</tr>
<tr>
<td>Com-A</td>
<td>TCCGCCCTGTTATATAAGTGCTACC</td>
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<tr>
<td>Primers for validation of the repaired strains</td>
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<tr>
<td>vC</td>
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<td>vA</td>
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<tr>
<td>INT-A</td>
<td>GATCCTTCTTTCTCTC</td>
</tr>
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2.3. Aerobic long-term survival

Bacterial cells at early log phase (OD_{600} = 0.3–0.5) were inoculated into 60 ml fresh 7H9-OADC-Tween 80 medium to OD_{600} = 0.016 in 500-ml Erlenmeyer flasks and incubated at 37 °C in a shaker at 220 rpm. After 20 days, the flask necks were covered with airtight plastic film to prevent evaporation, and the flasks were exposed to air for 2 min every 20 days. Cultures were sampled at regular time points to determine viability by CFU assay.

2.4. In vitro anaerobic model and recovery from dormancy

The rapid anaerobic dormancy (RAD) model was implemented by modifying the Wayne model.2 bacterial inoculants were aerobically grown at 37 °C in 7H9-OADC-Tween 80 medium with shaking. Bacterial cells in log phase (OD_{600} = 0.3–0.5) were diluted to OD_{600} = 0.20 using fresh medium and then inoculated to OD_{600} = 0.016. Cultures (7.1 ml) were aliquoted into 18-ml standard serum bottles at a culture-to-headspace ratio of 0.65. The bottle necks were sealed with butyl rubber stoppers and closed tightly with aluminum caps to avoid the leaking of air into the bottles. The cultures were allowed to grow rapidly by incubating them in a shaker at 240 rpm. Where indicated, methylene blue at a final concentration of 4.5 μg/ml was added during inoculation to indicate oxygen consumption. Bacterial viability was monitored at the time points indicated.

After 40 days in the RAD model, bacteria were exposed to oxygen and fresh medium in a series of dilutions and were allowed to resume growth as described previously.10 Bacterial growth was monitored daily for 5 days by plating bacteria onto 7H10-OADC agar. Growth rates were evaluated as fold increases in CFU numbers compared with those on Day 40.

2.5. Hypoxic and reductive model

Bacterial cells in early log phase (OD_{600} = 0.3–0.4) were diluted to OD_{600} = 0.20 using fresh 7H9-OADC-Tween 80 medium. Aliquots (4 ml) of the diluted cultures were loaded into 25-ml tubes. To produce hypoxic and reductive conditions, vitamin C was supplemented at a final concentration of 5 mM.9 After treatment for 24 h, INH (final concentration: 4 μg/ml) was added to kill replicating bacilli. Bacterial viability was measured by quantifying CFUs.

2.6. RNA extraction and microarray analysis

The wild type and Δ*Rv0195* strains were both grown in 7H9-OADC-Tween 80 medium under aerobic conditions and in the RAD model with methylene blue, which decolorized after 6.5 days. Bacterial cells were collected at OD_{600} = 1.3 for aerobic cultures or upon complete decolorization of methylene blue for cultures in the RAD model. Bacterial cells were incubated on ice for 1 min and then centrifuged at 12,000 g for 30 s. Freshly prepared chloroform/methanol (3:1, v/v) was added to the cell pellets at a ratio of 200 μl
per OD, vortexed for 20 s, and immediately mixed well with 5 vol Trizol (Takara, Dalian, China). Total RNA was extracted following the manufacturer’s instructions and inspected for RNA integration with an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, US). Verified total RNA was further purified using a RNeasy mini kit (Qiagen, Germany) and RNase-free DNase Set (Qiagen). DNA probes for all ORFs in *M. tuberculosis* were synthesized in situ on microarray matrices by Agilent Corp. DNA hybridization and signal scanning were carried out by the Biochip Company of Shanghai, China. Raw data were normalized with the Quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies); A cut-off value equivalent to a 3-fold change was used for selecting candidate genes.

The array data has been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE37973.

### 2.7. THP-1 cell infection model

The human acute monocytic leukemia cell line THP-1, obtained from the Institute of Biochemistry and Cell Biology, Shanghai, was grown in complete RPMI 1640 medium (Gibco-BRL) with 10% fetal calf serum. Cells at a concentration of $3 \times 10^5$/ml were induced to become macrophage-like cells by treating with 10 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. After removing the supernatant, the cells were infected with bacteria for 4 h at a multiplicity of infection (MOI) of 1, washed with incomplete RPMI 1640 medium and then incubated in fresh complete RPMI 1640 medium. Where indicated, a final concentration of 2 mM of sodium dodecyl sulfate was immediately added to the test wells post-infection. Total viable bacterial loads were assessed on days 0, 1 and 4 via lysis of the macrophages with 0.05% sodium dodecyl sulfate. Lysates were diluted in 7H9 broth and plated onto 7H10-OADC agar containing 0.05% Tween 80. An OD600 reading of 1.0 was determined to be 108 CFU/ml. Female C57BL/6 mice that were 5–6 weeks old were infected with $10^6$ CFU of bacteria via a lateral tail vein injection. On day 1 after infection, three mice from each group were sacrificed, and the number of implanted CFUs was determined both by PCR (Figure S1B) and sequencing (data not shown).

### 3.2. Rv0195 is inessential for growth and aerobic long-term survival

To assess the effect of Rv0195 on *in vitro* growth, bacteria were grown aerobically. CFU quantification showed that ΔRv0195 and Rv0195<sup>com</sup> grew along a similar curve compared with the wild type H37Rv (Figure 1A). After 80 days, the viabilities of the mutant and complemented bacilli were comparable to those of the wild type parental strain. These data demonstrate that Rv0195 is not required for bacterial growth and long-term survival under aerobicosis.

### 3.3. Rv0195 contributes to anaerobic survival

*M. tuberculosis* hypoxic/anaerobic dormancy has been studied using several *in vitro* models, particularly the Wayne model. In the present work, we modified the Wayne model to permit bacteria to consume the headspace oxygen quickly. Standard serum bottles with *M. tuberculosis* cultures were sealed with elastic butyl rubber stoppers and closed tightly with aluminum caps to avoid the leaking of air into the bottles. Bacteria grew rapidly at early stages with vigorous shaking but gradually entered dormancy concomitantly with the decrease in O2 in the headspace via respiration.

### 2.8. Mouse infections

Wild type H37Rv and ΔRv0195 were grown to early log phase at OD<sub>600</sub> = 0.3–0.4. The cultures were sonicated with a 2-mm-diameter probe at 80 W one time per milliliter of culture and then centrifuged at 200 g for 5 min to remove clumps. Cells were collected by centrifugation at 3000 g, adjusted to OD<sub>600</sub> = 0.20, and then diluted to OD<sub>600</sub> = 0.025 with basic Sauton’s medium containing 0.05% Tween 80. An OD<sub>600</sub> reading of 1.0 was determined to reflect $2 \times 10^8$ CFU/ml. Female C57BL/6 mice that were 5–6 weeks old were infected with $\sim 1 \times 10^6$ CFU of bacteria via a lateral tail vein injection. On day 1 after infection, three mice from each group were sacrificed, and the number of implanted CFUs was determined on 7H10 agar plate. At regular time points, three to four infected mice were sacrificed to determine CFU counts in lungs and spleens. Typically, half of each lung was used for CFU counts, and the other half was placed into formalin for histopathology.

### 2.9. Statistical analysis

To determine the statistical significance of differences observed for bacterial growth and survival, values were compared using a one-way ANOVA method.

### 3. Results

#### 3.1. Construction of the ΔRv0195 mutant and the complemented strain

To determine the role of the LuxR family transcription regulator Rv0195 in *M. tuberculosis*, a two-step allelic exchange method was used to construct an unmarked ORF deletion mutant (ΔRv0195) in H37Rv (Figure S1A). The mutant was obtained from double-crossover recombinants at a frequency of 23%. An integration plasmid with wild type Rv0195 was used to complement the mutant. The genotypes of ΔRv0195 and the complemented strain (Rv0195<sup>com</sup>) were confirmed both by PCR (Figure S1B) and sequencing (data not shown).
Upon the onset of anaerobiosis at day 7, no additional growth was observed, and the bacteria began to lose viability after that point. Wild type H37Rv lost its viability with a half-life of 10.5 days, which is similar to the half-life of 11 d observed with the Wayne model.3

Wild type H37Rv, ΔRv0195 and Rv0195com initially grew at similar rates in the RAD model (Figure 1B). In contrast to the survival patterns under aerobicosis, ΔRv0195 lost viability faster than the wild type strain upon entering anaerobiosis at the non-replicating phase 2 (NRP-2) stage.3,28 By 60 and 120 days, only 16% and 7% of initial ΔRv0195 bacterial numbers were recovered, respectively, in comparison to the wild type. Although Rv0195com did not completely restore the wild type phenotype, the transformation of Rv0195 into ΔRv0195 enhanced bacterial survival during anaerobiosis. These data indicate that Rv0195 is involved in M. tuberculosis survival during anaerobiosis.

3.4. Rv0195 mediates a rapid recovery from dormancy

When wild type H37Rv and ΔRv0195 survival in the RAD model were measured by determining CFU counts, we observed that the mutant bacilli took more time to form visible colonies on plates than the wild type. According to cultures examined on day 40, ΔRv0195 required an extra week to produce colonies (Figure 2A). As the incubation time under anaerobic conditions was extended, the delay required for colony formation became more apparent. However, no difference in colony growth was observed for wild type H37Rv, ΔRv0195 and Rv0195com when they were in the early stages of growth (<7 days) in the RAD model or under aerobic growth conditions (data not shown). Therefore, slow colony growth for ΔRv0195 occurred only after exposure to hypoxic or anaerobic conditions. The failure of Rv0195com to match wild type re-growth was likely caused by the burden of the integration plasmid, as the wild type parent containing the blank plasmid was also deficient in growth recovery after exposure to hypoxia and anaerobiosis.

We further quantitatively assessed the ability of anaerobic day 40 cultures to resume growth upon re-exposure to oxygen. After exposure to oxygen and fresh liquid medium, bacteria began to replicate within one day (Figure 2B). Similar to the phenotypes observed on solid plates, ΔRv0195 did not proliferate as well as wild type H37Rv. These results indicate that Rv0195 participates in the rapid recovery from dormancy.

3.5. Rv0195 contributes to survival from a hypoxic and reductive condition

As a reductive antioxidant and an essential nutrient involved in several vital cellular functions in humans, vitamin C can trigger growth arrest and dormancy in M. tuberculosis by scavenging O2 within a few hours, which is much more rapid than the Wayne model.2 Vitamin C was thus used to produce hypoxic and reductive conditions to evaluate the role of Rv0195 in dormancy. Bacterial growth was arrested in the presence of 5 mM vitamin C, which did not change the pH values of the cultures. After treatment with vitamin C, the viability of ΔRv0195 was slightly lower after 4 days compared to the wild type and the complemented strains (Figure 3).

The first-line antituberculosis drug INH specifically kills active M. tuberculosis, but it is not bactericidal against non-replicating dormant strains.3,4 We used INH to decrease the active subpopulation that existed in the in vitro model due to oxygen exposure as much as possible. In the presence of only INH, the survival of ΔRv0195 was comparable to wild type and complemented bacilli (Figure 3), suggesting that Rv0195 is not responsible for the antimicrobial activity of INH. The growth arrest with vitamin C treatment slightly promoted bacterial tolerance to INH for the wild type and complemented strains but not for ΔRv0195. After treatment with vitamin C and INH for 4 days and 7 days, ΔRv0195 viabilities were approximately 43% and 10% of the corresponding wild type viabilities, respectively. We observed that the CFUs/ml for bacteria treated with Vitamin C (VC) alone or INH alone were also less than 100 on day 7, and there was no difference between the three strains (data not shown). These data demonstrate that Rv0195 modulates M. tuberculosis survival under hypoxic and reductive conditions within a short period of time.

3.6. Rv0195 affects transcription of a series of genes

To investigate the genes affected by Rv0195, we compared the transcriptional profiles of the mutant and wild type strains via microarray hybridization analysis. Based on the impaired anaerobic/hypoxic dormancy of ΔRv0195, bacteria were harvested at the onset of anaerobiosis at the NRP-2 stage, as determined by the
oxygen indicator methylene blue.\textsuperscript{3} Transcriptome analysis revealed that the expression of approximately 180 genes was up- or down-regulated in ΔRv0195 (cut-off: ≥3-fold) in comparison to wild type H37Rv (Table 2, Table S1). These genes were classified into several categories spanning regulatory proteins to unknown conserved proteins. However, significant differences in the transcription levels of the DosR regulon genes were not observed for ΔRv0195 (Table S2); these genes were highly up-regulated upon exposure to anaerobiosis in the wild type strain.

3.7. Rv0195 controls virulence in THP-1 cells

The reduced dormancy phenotype of ΔRv0195 under in vitro anaerobic/hypoxic and reductive stress prompted us to evaluate the relevance of Rv0195 to virulence. To assess the intracellular proliferation of bacteria inside macrophages, the human THP-1 cell line pre-treated with PMA was infected with each genotype strain. Rv0195\textsuperscript{com} restored wild type growth levels in this model. The intracellular growth of ΔRv0195 was obviously arrested on day 4 compared with the wild type strain (Figure 4A). We next added 2 mM vitamin C into cultures to assess the viability of the mutant, as vitamin C can trigger the dormancy of intracellular M. tuberculosis.\textsuperscript{3} Similar to the results obtained with non-vitamin C conditions, the intracellular viability of ΔRv0195 was significantly impaired, exhibiting only 18% of the wild type level on day 4 (Figure 4B). These data demonstrate that Rv0195 is important for M. tuberculosis to maintain full virulence.

3.8. Rv0195 mediates pathogenicity in the C57BL/6 mouse

Based on the attenuated virulence of ΔRv0195 during macrophage infection, we next asked whether the mutant also experienced decreased pathogenicity in mice. C57BL/6 mice were infected with wild type H37Rv or ΔRv0195 via tail vein injections. Although almost identical amounts of bacteria were implanted, the effective loads (day 0) of ΔRv0195 in the lungs (Figure 5A) but not the spleens (Figure 5B) were lower than wild type H37Rv. During the early stages of infection, ΔRv0195 proliferated at the same rate as the wild type in the lungs, but ΔRv0195 CFU loads were always slightly lower than those of the wild type strain before 6 weeks. In spleens, ΔRv0195 CFU loads were lower than the wild type strain after infection for 1 week and were comparable to the wild type loads between 3 and 6 weeks.

Wild type H37Rv maintained viability in the lungs and spleens after infection for 10 weeks (Figure 5A, B). However, the viability of ΔRv0195 was significantly impaired during the late infection stage. After 10 weeks, 100-fold dilutions of ΔRv0195 bacilli isolated from lungs and spleens were not recovered on plates, even after cultivation for 2 months. Furthermore, histopathological analyses showed that the lesions in the lungs of mice infected with wild type bacilli appeared to be more severe than those caused by ΔRv0195, as less normal tissue was observed in lungs infected with wild type H37Rv (Figure 5C). These data indicate that Rv0195 is involved in M. tuberculosis virulence in mice.

4. Discussion

The mechanisms underlying M. tuberculosis dormancy still remain enigmatic, although the ability to enter dormancy is

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
Functional category & Gene number \\
\hline
Regulatory proteins & 7 \\
Virulence, detoxification, adaptation & 13 \\
Lipid metabolism & 11 \\
Cell wall and cell processes & 39 \\
Information pathways & 9 \\
Intermediary metabolism and respiration & 27 \\
Insertion sequences and phages & 11 \\
PEPPE family & 14 \\
Conserved hypothetical proteins & 48 \\
\hline
\end{tabular}
\caption{Functional categories of the genes affected by Rv0195.\textsuperscript{*}}
\end{table}

* Cut-off ≥3. For more information, see Table S1.
correlated with the two-component system DosR-DosS/DosT.\textsuperscript{7,9,10} In the present work, we demonstrate that the LuxR family regulator Rv0195 plays a vital role in \textit{M. tuberculosis} dormancy independent of the DosR system. Rv0195 is also important for bacterial virulence in human macrophage-like THP-1 cells and mouse tissues.

The physiological role of Rv0195 is dependent on the concentration of oxygen available for \textit{M. tuberculosis}. Under aerobic conditions, no difference was observed between the wild type, mutant, and complemented strains in terms of their long-term survival and growth rates. However, the \textit{Rv0195} mutant showed decreased survival under anaerobic conditions in our modified Wayne model. We also observed transcriptomic differences between anaerobic cultures of the mutant and wild type strains. Similar to Rv0195, the DosR system is responsible for the bacterial response to hypoxia/anaerobiosis as described in several studies.\textsuperscript{5–11} Recent unpublished work from our laboratory suggests that other signaling pathways, independent of Rv0195 and the DosR system, possibly exist in \textit{M. tuberculosis} to synergistically modulate bacterial responses to hypoxia/anaerobiosis. These findings suggest that the hypoxia/anaerobiosis response networks in \textit{M. tuberculosis} are more complicated than our present understanding indicates.

Bacterial viability analyses in our anaerobic/hypoxic models revealed that Rv0195 is important for \textit{M. tuberculosis} hypoxic/anaerobic dormancy. The deletion of \textit{Rv0195} resulted in impaired survival and recovery from anaerobic dormancy in the \textit{in vitro} RAD model. Dormancy defects were also previously observed in a DosR mutant, which exhibited 0.01% of wild type survival under anaerobic conditions and impaired recovery from dormancy.\textsuperscript{9} Furthermore, the dormancy-regulating role of \textit{Rv0195} was apparent in our hypoxic and reductive dormancy model when triggered by vitamin C.

\textit{Rv0195} acts as a regulator and affects the transcription profiles of many genes. LuxR family regulators usually sense AHL, typically in Gram-negative bacteria.\textsuperscript{18,19} \textit{M. tuberculosis} appears to be a non-AHL bacterium, as we did not measure any AHL when using a Gfp-based AHL sensor system\textsuperscript{20} (data not shown). Additionally, LuxI-like sequences were not found in the \textit{M. tuberculosis} genome. Therefore, the LuxR family member \textit{Rv0195} is unlikely to be an AHL sensor. A recent study reported that VpsT, a LuxR family member in \textit{M. tuberculosis}, is not an AHL sensor. A \textit{Rv0195} deletion mutant was previously observed in a DosR mutant, which exhibited 0.01% of wild type survival under anaerobic conditions and impaired recovery from dormancy.\textsuperscript{9} Recent unpublished work from our laboratory suggests that other signaling pathways, independent of Rv0195 and the DosR regulon, could synergistically modulate bacterial dormancy. Rv0195 modulates bacterial dormancy by a mechanism independent of the DosR regulon. The regulon is up-regulated upon exposure to hypoxia/NO/CO and other environmental cues.\textsuperscript{5–11} We also observed that the DosR regulon of the wild type strain was substantially up-regulated during anaerobic culture compared with aerobic culture. However, a comparison of the anaerobic transcription profiles of \textit{ΔRv0195} and wild type H37Rv showed no significant differences for the DosR regulon genes. Our findings and other studies\textsuperscript{11,12} suggest that \textit{M. tuberculosis} has evolved sophisticated regulatory networks with which bacteria enter, adapt to and survive dormancy.

We propose that the key genes mediating \textit{M. tuberculosis} dormancy also contribute to bacterial virulence in hosts. It is expected that \textit{M. tuberculosis} in hosts repeatedly cycles between dormancy and reactivation, and a dormancy-impaired bacterium is more easily eradicated by host tissues. For instance, the dormancy-impaired \textit{ΔRv0195} strain has attenuated virulence during infection of both human THP-1 cells and C57BL/6 mice. We observed that \textit{ΔRv0195} developed a dormancy-like phenotype triggered by some unknown mechanism, as the bacilli isolated from mouse lungs and spleens during the late phases of infection exhibited defects in colony formation. Furthermore, a mutant of the well-known dormancy regulator DosR showed a significant defect not only in hypoxic/anaerobic dormancy\textsuperscript{10} but also in virulence during infection of cells\textsuperscript{4} and granuloma-producing guinea pigs and rabbits.\textsuperscript{46,33,34} On the basis of these findings, we can assume that inhibition of key dormancy regulators in \textit{M. tuberculosis} could attenuate bacterial virulence in hosts with mechanisms that trigger bacterial dormancy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2013.04.005.

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Ethical approval: All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, issued and supervised by the National Institutes for Food and Drug Control of China. Animal protocols were approved by the Committee on the Ethics of Animal Experiments and the Institutional Animal Care and Use of the Beijing Tuberculosis and Thoracic Tumour Research Institute (permit number: M101230-1). All efforts were made to minimize animal numbers and suffering.

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