Tuberculosis 93 (2013) 425–431

Contents lists available at SciVerse ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MECHANISMS OF PATHOGENESIS

The LuxR family regulator Rv0195 modulates *Mycobacterium tuberculosis* dormancy and virulence

Haihong Fang [a,c], Dan Yu [a], Yuzhi Hong [a], Xiaodan Zhou [a], Chuanyou Li [b,**], Baolin Sun [a,*]

[a] Department of Microbiology and Immunology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China
[b] Department of Bacteriology and Immunology, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing 101149, China

ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form
10 April 2013
Accepted 16 April 2013

Keywords:
Tuberculosis
Mycobacterium tuberculosis
Dormancy
LuxR family transcription factor
Virulence

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 aerobiosis 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.

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*[1]; this population represents a large reservoir for active tuberculosis, as about 10% of latent infections ultimately develop into active tuberculosis.[2] It is thought that a certain proportion of infected individuals who remain asymptomatic harbor dormant bacilli[2] that are tolerant to antituberculosis drugs such as isoniazid (INH) and rifampin.[3,4] Dormancy of *M. tuberculosis* is thus a major cause for extended chemotherapeutic regimens and the wide epidemicity of tuberculosis.[5]

The current explanation for *M. tuberculosis* dormancy involves the DosR (originally designated DevR[6]) regulon, which consists of about 50 genes that are regulated by the two-component system DosR-DosS/T.[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,[5,7–10] NO[8] and CO,[11] and infections in macrophages,[12] mice[13] and guinea pigs,[14] which possibly triggers *M. tuberculosis* dormancy. However, the expression patterns of the DosR regulon are divergent for the H37Rv, H37Ra[15] and the W-Beijing lineage strains.[16] Disruption of *dosR* does not result in the rapid death of *M. tuberculosis* during hypoxia[7,9] and anaerobiosis.[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.[17] In Gram-negative proteobacteria, LuxR proteins usually modulate population density by sensing diffusible N-acyl homoserine lactone (AHL) molecules.[18,19] Typically, the LuxI and LuxR protein families are most commonly associated with AHL synthesis and the AHL signal response, respectively.[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.[21] LuxR proteins are divergent in terms of their primary

* Corresponding author. Tel./fax: +86 551 63606748.
** Corresponding author. Tel.: +86 10 89509366; fax: +86 10 69546819.
E-mail addresses: lichuanyou6688@hotmail.com (C. Li), sunb@ustc.edu.cn (B. Sun).
[c] Present address: Department of Microbiology, Anhui Medical University, Hefei, Anhui 230032, China.

1472-9792/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tube.2013.04.005

sequences and can exert diverse functions on various bacteria.[22] 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 *Hin*dIII, 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*I 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 competent cells by electroporation, and single-crossover and double-crossover colonies were screened following a method described previously.[25] Target mutants devoid of *Rv0195* were obtained from the double-crossovers and confirmed, first by PCR screening and then by DNA sequencing.

To complement the *Rv0195* mutation, a 1.17-kb fragment containing the ORF and the potential promoter 453 bp upstream of *Rv0195* was cloned into the pUC-Gm-INT vector, and the complemented plasmid pNG195^{com} was obtained. The complemented strain *Rv0195*^{com} was generated by transforming pNG195^{com} into Δ*Rv0195*. *Rv0195*^{com} was screened on 7H10 agar with gentamicin, and recombination of pNG195^{com} into the genome was confirmed by both PCR and DNA sequencing.

Table 1
Primers used in this study.

Primer	Sequence (5′ → 3′)	Description
Primers for suicide knockout plasmid construction		
5F-kS	CCCaagcttACGTTGCGTGGTGAAGTGG	*Hind*III
5F-A	TTGCACCCGGTCCGGACG	
3F-S	ccggaccgggtgcaaTTGATGGCCGGCGTCCGAC	Adaptor
5F-kA	CCCaagcttCCGAGGTGATTGCCCTGTCC	*Hind*III
Primers for validation of candidate Rv0195 mutants		
vS	GCTCTACATCGAGCTGTTGTACACG	Product: 2.8 kb for Δ*Rv0195*; 3.4 kb for wild type H37Rv
vA	GGCTTCGTTACAGAGGTTTTCCTG	
Primers for gene-complemented plasmid construction		
Com-S	CCaagcttAATGTATTGACGCATAGACG	*Eco*RI
Com-A	TCcccgggTTGCTATACATAATGGCTACCG	*Sma*I
Primers for validation of the repaired strains		
vcS	TGGCACCGGTGAATGTCATTTC	0.6 kb in *Rv0195*
vcA	CGGAGCTTCAGCCGCGTC	
INT-S	GCAGTCGCCCTAAAACAA	0.5 kb in the pUC-Gm-INT vector
INT-A	GCATCACTTCTTCCCGTA	

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} = 0.010 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.[3] 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} = 0.010. 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 aerobiotic 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 an 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 3×10^5/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 vitamin C 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 for CFU quantification in duplicate.

2.8. Mouse infections

Wild type H37Rv and Δ*Rv0195* were grown to early log phase at OD_{600} = 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 $OD_{600} = 0.20$, and then diluted to $OD_{600} = 0.025$ with basic Sauton's medium containing 0.05% Tween 80. An OD_{600} reading of 1.0 was determined to reflect 2×10^8 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.[26] 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[24,27] was used to construct an unmarked ORF deletion mutant (Δ*Rv0195*) 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 Δ*Rv0195* and the complemented strain (*Rv0195*^{com}) were confirmed 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 Δ*Rv0195* and *Rv0195*^{com} 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 aerobiosis.

3.3. Rv0195 contributes to anaerobic survival

M. tuberculosis hypoxic/anaerobic dormancy has been studied using several *in vitro* models, particularly the Wayne model.[3] 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 O_2 in the headspace via respiration

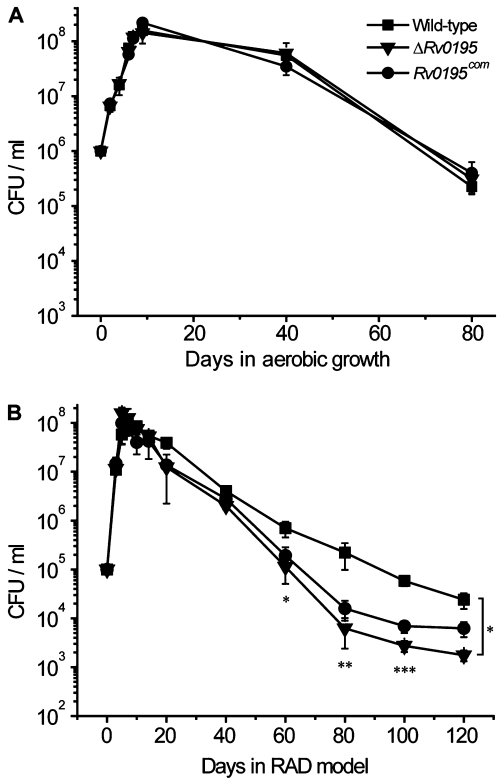

Figure 1. *Rv0195* modulates anaerobic but not aerobic long-term survival. (A) Bacterial growth and survival under aerobiotic conditions. Cells were grown in 500-ml conical flasks with 60 ml medium. (B) Bacterial growth and survival in the RAD model. Cells were grown in 18-ml serum bottles with a culture-to-headspace ratio of 0.65. Cultures were incubated on a shaker at 240 rpm. Bacterial viability was determined by recovery on solid medium. The data presented here are from one representative experiment. Each data point is the average of three independent cultures. *, $p < 0.02$; **, $p < 0.002$; ***, $p < 0.001$.

(Figure 1B). 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, Δ*Rv0195* and *Rv0195*^{com} initially grew at similar rates in the RAD model (Figure 1B). In contrast to the survival patterns under aerobiosis, Δ*Rv0195* 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 Δ*Rv0195* bacterial numbers were recovered, respectively, in comparison to the wild type. Although *Rv0195*^{com} did not completely restore the wild type phenotype, the transformation of *Rv0195* into Δ*Rv0195* 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 Δ*Rv0195* 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, Δ*Rv0195* required an extra week to produce colonies (Figure 2A). As the incubation time under anaerobiotic conditions was extended, the delay required for colony formation became more apparent. However, no difference in colony growth was observed for wild type H37Rv, Δ*Rv0195* and *Rv0195*^{com} 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 Δ*Rv0195* occurred only after exposure to hypoxic or anaerobic conditions. The failure of *Rv0195*^{com} 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.

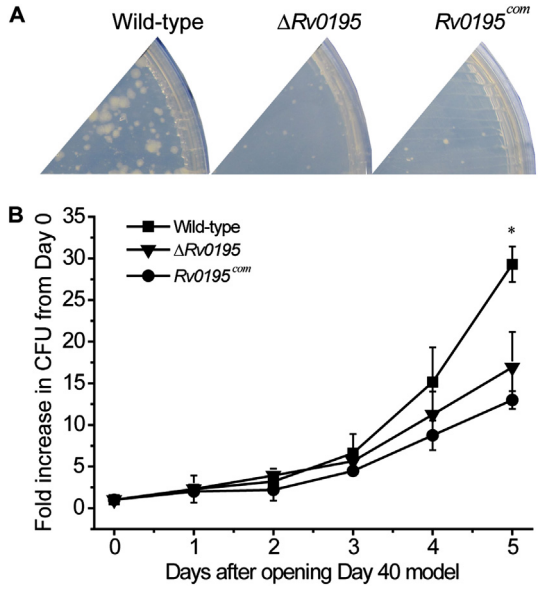

Figure 2. *Rv0195* contributes to rapid recovery from dormancy. (A) Colony formation during recovery from anaerobic dormancy. Anaerobic cultures in the RAD model were diluted and plated onto solid agar. (B) Re-growth rate of cultures during recovery from anaerobic dormancy. Day 40 cultures in the RAD model were diluted at a ratio of 1:1 and allowed to resume aerobic growth. The re-growth rate was calculated by comparing the fold increases in CFU numbers following methods described previously.[10] Each data point is the average of three independent anaerobic cultures. *, $p < 0.02$.

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, Δ*Rv0195* 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 O_2 within a few hours, which is much more rapid than the Wayne model.[9] 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 Δ*Rv0195* 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 Δ*Rv0195* 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 Δ*Rv0195*. After treatment with vitamin C and INH for 4 days and 7 days, Δ*Rv0195* 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 Δ*Rv0195*, bacteria were harvested at the onset of anaerobiosis at the NRP-2 stage, as determined by the

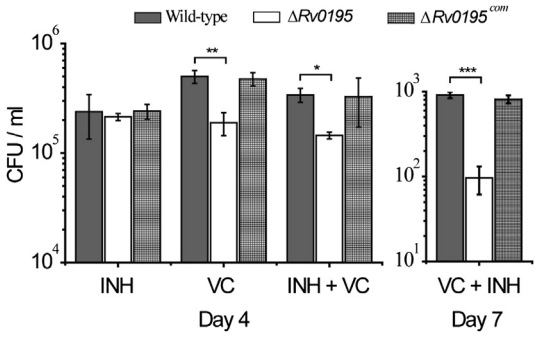

Figure 3. *Rv0195* is involved in survival under hypoxic and reductive conditions. Bacterial cells were inoculated into fresh liquid medium to ~1 × 10^6 CFU/ml and immediately treated with 2 mM vitamin C where appropriate. After treatment for 24 h, INH was added to a final concentration of 4 μg/ml. Each data point is the average of three independent cultures. *, $p < 0.006$; **, $p < 0.002$; ***, $p < 0.0001$.

oxygen indicator methylene blue.[3] Transcriptome analysis revealed that the expression of approximately 180 genes was up- or down-regulated in Δ*Rv0195* (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 Δ*Rv0195* (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 Δ*Rv0195* 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*^{com} restored wild type growth levels in this model. The intracellular growth of Δ*Rv0195* 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*.[9] Similar to the results obtained with non-vitamin C conditions, the intracellular viability of Δ*Rv0195* 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 Δ*Rv0195* 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 Δ*Rv0195* via tail vein injections. Although almost identical amounts of bacteria were implanted, the effective loads (day 0) of Δ*Rv0195* in the lungs (Figure 5A) but not the spleens (Figure 5B) were lower than wild type H37Rv. During the early stages of infection, Δ*Rv0195* proliferated at the same rate as the wild type in the lungs, but Δ*Rv0195* CFU loads were always slightly lower than those of the wild type strain before 6 weeks. In spleens, Δ*Rv0195* 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 Δ*Rv0195* was significantly impaired during the late infection stage. After 10 weeks, 100-fold dilutions of Δ*Rv0195* 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 Δ*Rv0195*, 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.

Table 2
Functional categories of the genes affected by *Rv0195*.*

Functional category	Gene number
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
PE/PPE family	14
Conserved hypothetical proteins	48

* Cut-off ≥3. For more information, see Table S1.

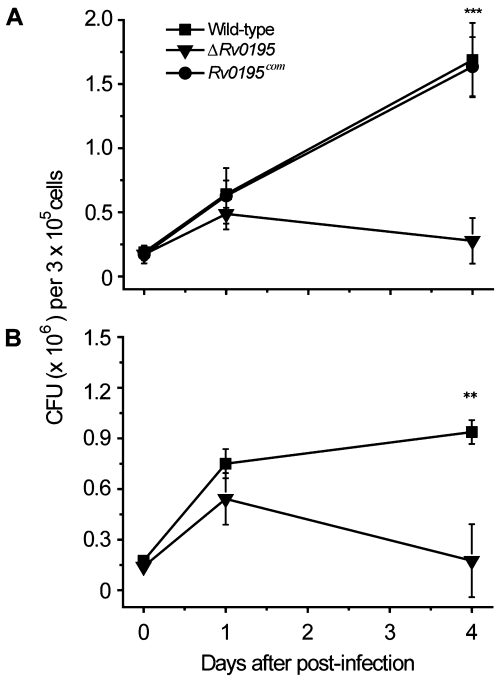

Figure 4. *Rv0195* controls bacterial virulence in human THP-1 cells. Cells were infected with *M. tuberculosis* at an MOI of 1. (A) Bacterial proliferation within macrophages. Each data point is the average of six independent cultures from two independent experiments. ***, $p < 0.001$. (B) Bacteriostasis within vitamin C-treated macrophages. THP-1 cells were treated with 2 mM vitamin C immediately after bacterial infection. **, $p < 0.007$.

4. Discussion

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

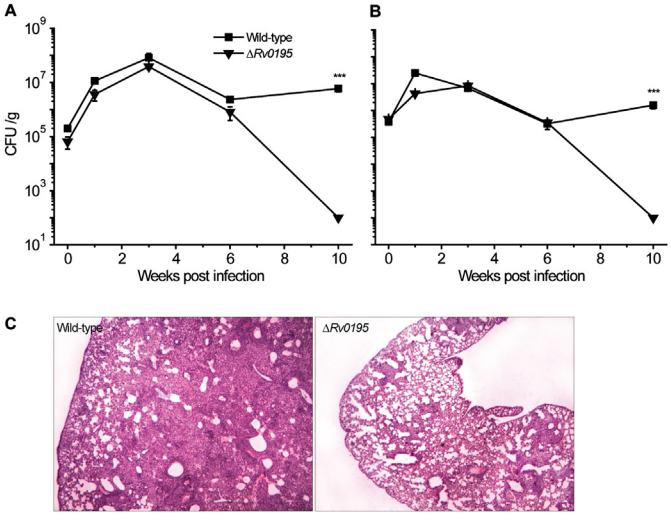

Figure 5. *Rv0195* mediates pathogenicity in mouse tissues. Approximately 1×10^6 bacterial CFUs were implanted into each mouse via tail vein injection. Bacterial loads were determined for lungs (A) and spleens (B). Each data point is the average of three to four independent mice. **, $p < 0.004$. (C) Histopathology of lungs from C57BL/6 mice. By week 6, lesions were larger and denser in wild type-infected mouse lungs compared to lesions from Δ*Rv0195*-infected mouse lungs, in which more normal lung tissue was preserved. Magnification ×40.

correlated with the two-component system DosR-DosS/DosT.[7,9,10] In the present work, we demonstrate that the LuxR family regulator Rv0195 plays a vital role in *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 *M. tuberculosis*. Under aerobiotic 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 *Rv0195* mutant showed decreased survival under anaerobiotic 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.[5–11] Recent unpublished work from our laboratory suggests that other signaling pathways, independent of Rv0195 and the DosR system, possibly exist in *M. tuberculosis* to synergistically modulate bacterial responses to hypoxia/anaerobiosis. These findings suggest that the hypoxia/anaerobiosis response networks in *M. tuberculosis* are more complicated than our present understanding indicates.

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

Rv0195 acts as a regulator and affects the transcription profiles of many genes. LuxR family regulators usually sense AHL, typically in Gram-negative bacteria.[18,19] *M. tuberculosis* appears to be a non-AHL bacterium, as we did not measure any AHL when using a Gfp-based AHL sensor system[29] (data not shown). Additionally, *LuxI*-like sequences were not found in the *M. tuberculosis* genome. Therefore, the LuxR family member Rv0195 is unlikely to be an AHL sensor. A recent study reported that VpsT, a LuxR family member in *Vibrio cholera*, acts as a c-di-GMP-sensing global switch to control extracellular matrix production and motility.[30] The amino acid sequences of Rv0195 and VpsT have a similarity of 48% (Figure S2), suggesting that Rv0195 may be a potential c-di-GMP receptor. Unfortunately, we failed to obtain soluble Rv0195 protein for further biochemical assays when it was heterologously expressed in *E. coli* (data not shown). Interestingly, a comparison of the anaerobic transcriptomes for Δ*Rv0195* and Δ*dgc* (a dormancy-enhanced mutant of the c-di-GMP synthase, our unpublished work) revealed that 18 genes with differential expression that overlapped (Table S3). These genes exhibited opposite transcriptional patterns in the two mutants. These data suggest that the *Rv0195*-mediated network may interact with other dormancy-related signaling pathways to regulate *M. tuberculosis* 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.[5–12] 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 Δ*Rv0195* and wild type H37Rv showed no significant differences for the DosR regulon genes. Our findings and other studies[31,32] suggest that *M. tuberculosis* has evolved sophisticated regulatory networks with which bacteria enter, adapt to and survive dormancy.

We propose that the key genes mediating *M. tuberculosis* dormancy also contribute to bacterial virulence in hosts. It is expected that *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 Δ*Rv0195* strain has attenuated virulence during infection of both human THP-1 cells and C57BL/6 mice. We observed that Δ*Rv0195* 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[7,10] but also in virulence during infection of cells[4] and granuloma-producing guinea pigs and rabbits.[26,33,34] On the basis of these findings, we can assume that inhibition of key dormancy regulators in *M. tuberculosis* could attenuate bacterial virulence in hosts with mechanisms that trigger bacterial dormancy.

Acknowledgments

We thank Tanya Parish (Queen Mary University of London) for the generous gift of the vectors for gene knockout and complementation. We are grateful to Weicong Ren, Yi Liu and Miao Tian for their technical assistance.

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.

Funding: This study was supported by grants from the Eleventh Five-Year National S&T Major Project of China (2008ZX10003006) (BS) and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-164) (BS). The funders had no role in experimental design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: None declared.

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.

References

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. *J Am Med Assoc* 1999;**282**:677–86.
2. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* 2001;**19**:93–129. http://dx.doi.org/10.1146/annurev.immunol.19.1.93.
3. Wayne LG, Hayes LG. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* 1996;**64**:2062–9.
4. Hussain S, Malik M, Shi L, Gennaro ML, Drlica K. *In vitro* model of mycobacterial growth arrest using nitric oxide with limited air. *Antimicrobial Agents Chemother* 2009;**53**:157–61. http://dx.doi.org/10.1128/AAC.00442-08.
5. Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK. Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding alpha-crystallin. *Proc Natl Acad Sci USA* 2001;**98**:7534–9. http://dx.doi.org/10.1073/pnas.121172498.
6. Dasgupta N, Kapur V, Singh KK, Das TK, Sachdeva S, Jyothisri K, Tyagi JS. Characterization of a two-component system, *devR-devS*, of Mycobacterium tuberculosis. *Tuber Lung Dis* 2000;**80**:141–59.

7. Rustad TR, Harrell MI, Liao R, Sherman DR. The enduring hypoxic response of Mycobacterium tuberculosis. *PloS One* 2008;**3**:e1502. http://dx.doi.org/10.1371/journal.pone.0001502.
8. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* 2003;**198**:705–13. http://dx.doi.org/10.1084/jem.20030205.
9. Taneja NK, Dhingra S, Mittal A, Naresh M, Tyagi JS. *Mycobacterium tuberculosis* transcriptional adaptation, growth arrest and dormancy phenotype development is triggered by vitamin C. *PloS One* 2010;**5**:e10860. http://dx.doi.org/10.1371/journal.pone.0010860.
10. Leistikow RL, Morton RA, Bartek IL, Frimpong I, Wagner K, Voskuil MI. The *Mycobacterium tuberculosis* DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy. *J Bacteriol* 2010;**192**:1662–70. http://dx.doi.org/10.1128/JB.00926-09.
11. Kumar A, Deshane JS, Crossman DK, Bolisetty S, Yan BS, Kramnik I, Agarwal A, Steyn AJ. Heme oxygenase-1-derived carbon monoxide induces the *Mycobacterium tuberculosis* dormancy regulon. *J Biol Chem* 2008;**283**:18032–9. http://dx.doi.org/10.1074/jbc.M802274200.
12. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* 2003;**198**:693–704. http://dx.doi.org/10.1084/jem.20030846.
13. Karakousis PC, Yoshimatsu T, Lamichhane G, Woolwine SC, Nuermberger EL, Grosset J, Bishai WR. Dormancy phenotype displayed by extracellular *Mycobacterium tuberculosis* within artificial granulomas in mice. *J Exp Med* 2004;**200**:647–57. http://dx.doi.org/10.1084/jem.20040646.
14. Sharma D, Bose A, Shakila H, Das TK, Tyagi JS, Ramanathan VD. Expression of mycobacterial cell division protein, FtsZ, and dormancy proteins, DevR and Acr, within lung granulomas throughout guinea pig infection. *FEMS Immunol Med Microbiol* 2006;**48**:329–36. http://dx.doi.org/10.1111/j.1574-695X.2006.00160.x.
15. Malhotra V, Tyagi JS, Clark-Curtiss JE. DevR-mediated adaptive response in *Mycobacterium tuberculosis* H37Ra: links to asparagine metabolism. *Tuberculosis* 2009;**89**:169–74.
16. Minch K, Rustad T, Sherman DR. *Mycobacterium tuberculosis* growth following aerobic expression of the DosR regulon. *PloS One* 2012;**7**:e35935. http://dx.doi.org/10.1371/journal.pone.0035935.
17. Pompeani AJ, Irgon JJ, Berger MF, Bulyk ML, Wingreen NS, Bassler BL. The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol Microbiol* 2008;**70**:76–88. http://dx.doi.org/10.1111/j.1365-2958.2008.06389.x.
18. Withers H, Swift S, Williams P. Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. *Curr Opin Microbiol* 2001;**4**:186–93.
19. Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* 2001;**25**:365–404.
20. Galloway WR, Hodgkinson JT, Bowden SD, Welch M, Spring DR. Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev* 2011;**111**:28–67. http://dx.doi.org/10.1021/cr100109t.
21. Subramoni S, Venturi V. LuxR-family 'solos': bachelor sensors/regulators of signalling molecules. *Microbiology* 2009;**155**:1377–85. http://dx.doi.org/10.1099/mic.0.026849-0.
22. Zeng LR, Xie JP. Molecular basis underlying LuxR family transcription factors and function diversity and implications for novel antibiotic drug targets. *J Cell Biochem* 2011;**112**:3079–84. http://dx.doi.org/10.1002/jcb.23262.
23. Agarwal N, Lamichhane G, Gupta R, Nolan S, Bishai WR. Cyclic AMP intoxication of macrophages by a *Mycobacterium tuberculosis* adenylate cyclase. *Nature* 2009;**460**:98–102. http://dx.doi.org/10.1038/nature08123.
24. Parish T, Stoker NG. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis tlyA plcABC* mutant by gene replacement. *Microbiology* 2000;**146**:1969–75.
25. Parish T, Stoker NG. *Mycobacterium tuberculosis protocols*. Totowa, NJ: Humana Press Inc.; 2000. p. 77–92.
26. Converse PJ, Karakousis PC, Klinkenberg LG, Kesavan AK, Ly LH, Allen SS, Grosset JH, Jain SK, Lamichhane G, Manabe YC, McMurray DN, Nuermberger EL, Bishai WR. Role of the *dosR-dosS* two-component regulatory system in *Mycobacterium tuberculosis* virulence in three animal models. *Infect Immun* 2009;**77**:1230–7. http://dx.doi.org/10.1128/IAI.01117-08.
27. Parish T, Smith DA, Kendall S, Casali W, Bancroft GJ, Stoker NG. Deletion of two-component regulatory systems increases the virulence of *Mycobacterium tuberculosis*. *Infect Immun* 2003;**71**:1134–40.
28. Wayne LG, Sohaskey CD. Nonreplicating persistence of Mycobacterium tuberculosis. *Annu Rev Microbiol* 2001;**55**:139–63. http://dx.doi.org/10.1146/annurev.micro.55.1.139.
29. Andersen JB, Heydorn A, Hentzer M, Eberl L, Geisenberger O, Christensen BB, Molin S, Givskov M. GFP-based N-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl Environ Microbiol* 2001;**67**:575–85. http://dx.doi.org/10.1128/AEM.67.2.575-585.2001.
30. Krasteva PV, Fong JCN, Shikuma NJ, Beyhan S, Navarro MVAS, Yildiz FH, Sondermann H. *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. *Science* 2010;**327**:866–8.
31. Iona E, Pardini M, Gagliardi MG, Colone M, Stringaro AR, Teloni R, Brunori L, Nisini R, Fattorini L, Giannoni F. Infection of human THP-1 cells with dormant *Mycobacterium tuberculosis*. *Microbes Infect* 2012;**14**:959–67.
32. Kapoor N, Pawar S, Sirakova TD, Deb C, Warren WL, Kolattukudy PE. Human granuloma *in vitro* model, for TB dormancy and resuscitation. *PloS One* 2013;**8**:e53657.
33. Lenaerts AJ, Hoff D, Aly S, Ehlers S, Andries K, Cantarero L, Orme IM, Basaraba RJ. Location of persisting mycobacteria in a guinea pig model of tuberculosis revealed by R207910. *Antimicrobial Agents Chemother* 2007;**51**:3338–45. http://dx.doi.org/10.1128/AAC.00276-07.
34. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Eum SY, Taylor K, Klein E, Manjunatha U, Gonzales J, Lee EG, Park SK, Raleigh JA, Cho SN, McMurray DN, Flynn JL, Barry CE. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect Immun* 2008;**76**:2333–40. http://dx.doi.org/10.1128/IAI.01515-07.