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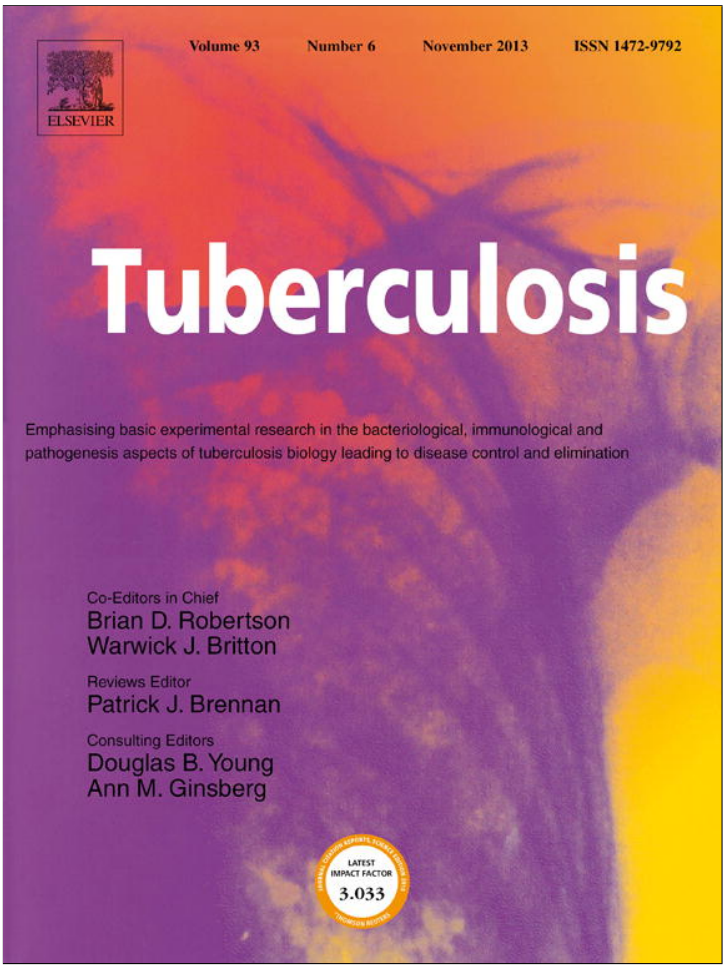

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MECHANISMS OF PATHOGENESIS

Cyclic di-GMP mediates *Mycobacterium tuberculosis* dormancy and pathogenecity

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

[a] 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 Tumour Research Institute, Beijing 101149, China

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SUMMARY

Dormancy of *Mycobacterium tuberculosis* is likely to be a major cause of extended chemotherapeutic regimens and wide prevalence of tuberculosis. The molecular mechanisms underlying *M. tuberculosis* dormancy are not well understood. In this study, single-copy genes responsible for synthesis (*dgc*) and degradation (*pde*) of the ubiquitous bacterial second messenger, cyclic di-GMP (c-di-GMP), were deleted in the virulent *M. tuberculosis* strain H37Rv to generate dgc^{mut} and Δ*pde*, respectively. Under aerobic growth conditions, the two mutants and wild-type cells showed similar phenotypes. However, dgc^{mut} and Δ*pde* exhibited increased and reduced dormancy, respectively, in both anaerobiosis-triggered and vitamin C-triggered *in vitro* dormancy models, as determined by survival and growth recovery from dormancy. The transcriptomes of aerobic cultures of dgc^{mut} and wild-type H37Rv exhibited no difference, whereas those of anaerobic cultures showed a significant difference with 61 genes that are not a part of the *dosR* regulon. Furthermore, Δ*pde* but not dgc^{mut} showed decreased infectivity with human THP-1 cells. Δ*pde* also showed attenuated pathogenicity in a C57BL/6 mouse infection model. These findings are explained by c-di-GMP-mediated signaling negatively regulating *M. tuberculosis* dormancy and pathogenicity.

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1. Introduction

Tuberculosis (TB) continues to prevail as a major cause of human infectious mortality around the world. The causative agent, *Mycobacterium tuberculosis*, latently infects nearly one-third of the world population, with about one in ten latent infections eventually progressing to acute disease [1]. Latency plays a key role in enabling this slow-growing bacterium to achieve worldwide prevalence [2]. Clinical latency is ascribed to the tendency of *M. tuberculosis* to enter a dormant state upon exposure to catastrophic stresses, such as anaerobiosis and host immunity. Dormant bacilli have a decreased metabolic status that renders *M. tuberculosis* less susceptible to anti-tuberculosis drugs [3–5]. Identifying the mechanisms used by *M. tuberculosis* to enter into, survive, and reactivate from dormancy may provide clues for discovery of new anti-TB drugs and vaccines.

The current explanation for *M. tuberculosis* dormancy focuses on the two-component DosR(DevR)-DosS/DosT system. The DosR regulon is induced by a variety of conditions that include hypoxia [2,6–9], exposure to NO [10] or CO [11], and the early stages of infection of macrophages [12], dendritic cells [13], mice [14] and guinea pigs [15]. Changes in O_2/NO/CO and/or redox balance are thought to be sensed by DosT and/or DosS [16–20]. Upon activation with these changes, DosT and DosS deliver activating phosphate groups to DosR, which then up-regulates the expression of about 50 genes in the DosR regulon [9]. The transcription of *dosR* is autoregulated [21], and a few genes of the regulon are also regulated by the transcriptional regulator PhoP [22]. Of the potential dormancy-inducing cues, hypoxia is the best-studied, as both *in vitro* [4,6–8] and *in vivo* [6,23] models are available. However, several studies found that the DosR regulon is activated at an early stage under moderate hypoxia and is not responsible for the adaptation necessary to survive during hypoxia [2,6,24]. Consequently, other mechanism(s) must exist that, together with the DosR system, are used by *M. tuberculosis* to enter into, survive, and reactivate from dormancy.

In the present work we examined the possibility that bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) participates in *M. tuberculosis* dormancy and pathogenecity. C-di-GMP is a ubiquitous bacterial second messenger that modulates many important biological processes including virulence (for reviews, see [25,26]). C-di-GMP is synthesized by diguanylate cyclases (Dgc) that have a

* 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).

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conserved GGDEF domain, and it is broken down by phosphodiesterases (Pde) containing a conserved EAL or HD-GYP domain [25,26]. *M. tuberculosis* encodes a copy of *dgc* (*Rv1354c*) and *pde* (*Rv1357c*). Rv1354c habours GAF, GGDEF and EAL domains in tandem, and Rv1357c contains only an EAL domain [27]. The expected c-di-GMP synthesizing and degrading activities of the two enzymes have been established *in vitro* by using refolded proteins [27]. In *Mycobacterium smegmatis*, a non-pathogenic relative of *M. tuberculosis*, deletion of *dgc* decreases bacterial long-term survival during aerobic nutritional starvation [28]. Placement of *M. tuberculosis dgc* into the mutant restores bacterial survival [27]. We generated the *dgc* and *pde* mutants in *M. tuberculosis*, and compared their dormancy and virulence with the wild-type strain by using *in vitro* and *in vivo* models. We show that c-di-GMP signaling in virulent *M. tuberculosis* does not affect aerobic long-term survival, but it negatively modulates bacterial anaerobic dormancy by a mechanism that is independent of the DosR system. Furthermore, c-di-GMP is responsible for the negative regulation of bacterial virulence with infections of human macrophage-like cells and mice.

2. Materials and methods

2.1. Growth and construction of bacterial strains

M. tuberculosis virulent strain H37Rv was grown at 37 °C in Middlebrook 7H9 medium containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) supplement (Difco Laboratories) and 0.05% (wt/vol) Tween 80 or on Middlebrook 7H10-OADC agar. The deletion-delivery suicide vectors of *dgc* and *pde* were constructed by polymerase chain reaction (PCR) with appropriate primers (Table S1) using the p1NIL and pGOAL19 vectors following methods described previously [29]. Single and double crossovers were screened as described [29]. The mutants of *dgc* and *pde* were obtained by deletions of the GGDEF-encoding domain in *dgc* and the whole open reading frame (ORF) of *pde*, respectively. The mutants were confirmed by a combination of PCR and DNA sequence determination.

To repair the *dgc* mutation, a 2.18 kb DNA fragment containing the entire *dgc* open reading frame (ORF) and a 308 bp potential promoter of the *dgc* operon were cloned into an integrating plasmid pUC-Gm-INT [29]. For *pde* complementation, a 1.23 kb fragment containing the *pde* ORF and 262 bp upstream of it was used. The constructs were introduced into the dgc^{mut} and Δ*pde* mutants by bacterial transformation [30] to generate the repaired/complemented strains dgc^{rep} and pde^{com}, respectively.

2.2. In vitro rapid anaerobic dormancy (RAD) model

The wild-type, mutant and complemented strains were grown in 7H9-OADC-Tween 80 medium to an $OD_{600} = 0.3 - 0.5$, adjusted to OD = 0.20 with fresh medium, and then diluted into fresh medium to OD = 0.01. The RAD model experiments were performed using 18-ml (or 150-ml) standard serum bottles containing 7.1 ml (or 60 ml) culture at a culture-to-headspace ratio of 0.65. The bottles were sealed with butyl rubber stoppers and closed tightly with aluminum caps. Cultures were grown by shaking at 240 rpm. The oxygen-sensitive indicator methylene blue was added during inoculation at a final concentration of 4.5 μg/ml, where appropriate. The viability of cultures at various time points was monitored via colony-forming unit (CFU) determination.

2.3. Isolation and detection of intracellular c-di-GMP

Wild-type H37Rv and dgc^{mut} were grown on 7H10-OADC agar or in the RAD model. Cells were collected after 3 weeks for the aerobic cultures and at the onset of anaerobiosis for the anaerobic cultures. Cells at wet weights of 0.2–0.5 g were used for extraction of total nucleotides by using a method with $HClO_4$ as described previously [31]. Components in samples were assayed by using liquid chromatography–mass spectrometry (LC–MS, LTQ Orbitrap XL). Samples (20 μl) were loaded onto a C-18 column (4.6 × 150 mm, Agilent Eclipse XDB-C18) and separated by reverse-phase HPLC (Agilent 1200). Samples were eluted with a buffer [20 mM ammonium acetate (pH 5.9) with 0.05% (v/v) methanol] at 0.2 ml/min. The buffer was diluted from 100% to 80% by using methanol during 5–20 min. Nucleotides were monitored at 254 nm and determined by mass spectrometry. The c-di-GMP content was calculated from calibration using pure c-di-GMP (BIOLOG Life Science Institute, Germany).

2.4. Recovery from dormancy

After 40 days of dormancy, bacteria were exposed to oxygen and fresh medium (diluted 1:1) and were allowed to resume growth, following the methods described by Leistikow et al. [7]. Bacterial growth was measured daily for 5 days by plating bacteria onto 7H10-OADC agar. Growth rates were described as the fold increases in the number of CFU relative to the number of bacteria at day 40.

2.5. Aerobic growth and survival

Bacteria were inoculated as in the RAD model and grown for 80 days in 500 ml Erlenmyer flasks with 60 ml culture by shaking at 220 rpm. After 20 days, the flasks were closed by airtight plastic film to prevent evaporation. The cultures were exposed to air every 20 days.

2.6. Hypoxic and reductive model

The model was conducted as described previously [32]. Briefly, *M. tuberculosis* strains at log phases ($OD_{600} = 0.3 - 0.4$) 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, and treated with vitamin C (final 5 mM) or sodium nitroprusside (SNP, 100 mM). After treatment for 24 h, isoniazide (INH) (4 μg/ml) was added to kill active bacilli.

2.7. RNA extraction and microarray hybridization analysis

Cells of wild-type H37Rv and dgc^{mut} were collected at $OD_{600} = 1.3$ for aerobic cultures or upon the beginning of complete decolorisation of methylene blue for the anaerobic cultures in the RAD model. Total RNA was extracted by a Trizol method (Takara, Dalian, China). RNA purification, cDNA synthesis and labeling, hybridization, and scanning were conducted by the Biochip Company of Shanghai, China. Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies). Two independent biological replicates were included for dgc^{mut}.

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

2.8. THP-1 cell infection model

Human THP-1 cell lines were grown in RPMI 1640 medium (Gibco-BRL) with 10% fetal calf serum. Cultures of the wild-type, mutant and complemented strains were maintained at concentrations of 3×10^5 cells/ml and were induced into a macrophage-like state by treatment with 10 nM of 12-O-tetradecanoylphorbol-13-acetate over 24 h. After removing the suspension, the adherent cells were infected with *M. tuberculosis* for 4 h at a multiplicity of infection (MOI) of 1. The contents of the culture plate

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wells were subsequently washed with incomplete RPMI 1640 medium and incubated in fresh complete RPMI 1640 medium. Cell treatment and bacterial viability assay were as described by Taneja et al. [6]. Where indicated, vitamin C was added to a final concentration of 2 mM immediately after bacterial infection.

2.9. Mouse infection

Cultures of wild-type H37Rv and Δ*pde* at early log phase were sonicated at 80 W for several times and centrifuged at 200 *g* to remove clumps. The cell suspension was prepared as in the RAD model and diluted to $OD_{600} = 0.025$ with basic Sauton's medium containing 0.05% Tween-80. Cultures having an OD_{600} of 1.0 contained approximately 2×10^8 CFU/ml. Female C57BL/6 mice (5–6 week old) were infected with 1×10^6 CFU via tail vein injection. At weeks 1, 3, 6 and 10, three to four mice from each group were sacrificed for lung and spleen CFU determination. The CFUs on day 1 after infection were referred to as the implanted bacterial loads. CFU counts and histopathology were conducted as described [23].

2.10. Statistical analysis

Statistical significance of values was compared using a one-way ANOVA method. The *P* values are indicated in each figure legend.

3. Results

3.1. Generation of dgc and pde mutants and complemented strains

To examine the role of the c-di-GMP signal transduction pathway in *M. tuberculosis*, a two-step allelic exchange method was used to construct unmarked deletion mutants of *dgc* and *pde* in virulent H37Rv (Figure 1A). The shortened *dgc* mutant (dgc^{mut}) was generated by deletion of the conserved GGDEF domain (R^{196}–H^{315}) that is responsible for c-di-GMP synthesis (20 mutants were obtained from 80 double-crossover colonies). The deletion did not change the reading frames of the other encoding sequence in *Rv1354c* (Figure 1B), thereby avoiding polar effects in the probable *Rv1353c-dgc-Rv1355c-Rv1356c* operon. The shortened peptide is non-functional due to a damaged structure [27]. The *pde* mutant (Δ*pde*) was generated by deletion of its ORF (12 mutants being obtained from 36 double-crossover colonies). dgc^{mut}, Δ*pde* and their corresponding repaired/complemented strains dgc^{rep} and pde^{com} were confirmed by PCR and DNA sequence determination.

3.2. dgc is responsible for intracellular c-di-GMP synthesis

In vitro enzymatic assays have established that *M. tuberculosis* Dgc and Pde have c-di-GMP-synthesizing and -degrading activity, respectively [27]. To determine whether the *dgc* mutation results in elimination of intracellular c-di-GMP in *M. tuberculosis*, we measured the compound levels in dgc^{mut} compared with wild-type H37Rv. For the wild-type cultures collected at onset of anaerobiosis, the c-di-GMP level in cells was about 0.1 pmol per gram of wet bacteria (Figure 2). For the aerobic cultures from agar plates, the c-di-GMP content in the wild-type parent was about 0.5 pmol per gram of wet bacteria. No c-di-GMP was detected in dgc^{mut} under either aerobic or anaerobic conditions (limit of detection was about 1 f mol per gram of wet bacteria). The accurate c-di-GMP levels at different stages and in different strains need further tests, as c-di-GMP is not very stable in the extracts prepared by the perchloric acid method. Nevertheless, these data indicated that *dgc* is the only gene responsible for c-di-GMP synthesis in *M. tuberculosis*.

3.3. Rapid anaerobic dormancy (RAD) model

In this model, standard serum bottles, commonly used for anaerobic microbial growth, were used to culture *M. tuberculosis*. Bacteria were allowed to grow rapidly by shaking on a rotary shaker rather than with gentle stirring as described for the Wayne model [3]. Cells gradually entered into dormancy when the oxygen in the headspace was consumed via respiration. Wild-type H37Rv lost its viability rapidly after the onset of anaerobiosis (day 7) with a half-life of 10.5 d (Figure 3A), similar to the half-life of 11 d reported for the Wayne model [3]. The bacterial survival described here was slightly different from that of another modified RAD model described previously [7]. In that model, *M. tuberculosis* survived much longer (no loss of viability was observed until after 40 d).

3.4. Absence of dgc alters anaerobic transcriptional profile

To provide clues for guiding experiments about potential physiological phenotypes regulated by c-di-GMP, microarray hybridization analyses were performed to compare the transcriptional profiles of dgc^{mut} and wild-type H37Rv. We first compared the transcriptomes of their aerobic cultures at late-log phase ($OD_{600} = 1.3$), as *dgc* (c-di-GMP) is essential for aerobic long-term survival in the related species *M. smegmatis* [28]. Unexpectedly, no difference was observed (data not shown).

Since *M. tuberculosis* tends to enter a dormant state when it is exposed to anaerobic conditions, we next asked whether c-di-GMP signaling plays a role under hypoxia/anaerobiosis. Bacteria were collected for RNA preparation at the onset of anaerobiosis in the RAD model. Transcriptome analysis showed that 61 genes were up- or down-regulated in dgc^{mut} (cut-off $\geq$ 3-fold) in comparison with wild-type H37Rv (Table S2). Most of the down-regulated genes appeared to be involved in cell metabolism. Interestingly, the principal sigma factor-encoding gene, *sigA*, was also down-regulated, whereas the survival-enhancing gene *eis* [30,33] was up-regulated. No obvious difference was observed for the DosR regulon genes [9,10,12]. These data suggested that c-di-GMP signaling may be involved in previously unknown behavior of *M. tuberculosis* under hypoxia/anaerobiosis.

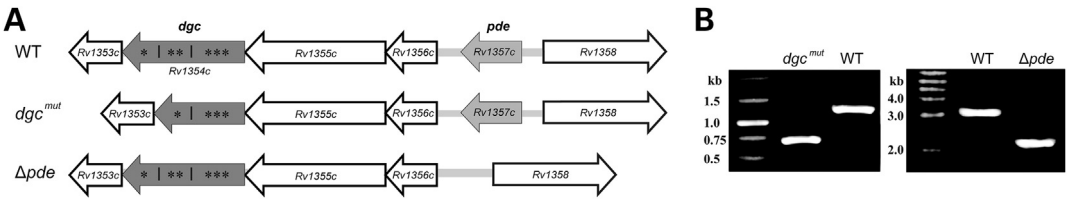

Figure 1. Construction of the dgc^{mut} and Δ*pde* mutants. (A) Gene arrangements in the wild-type and mutant strains. Arrows indicate the approximate positions and directions of genes in the H37Rv genome. *, the GAF domain-encoding sequence in DGC; **, the GGDEF domain-encoding sequence; ***, the EAL domain-encoding sequence. (B) PCR screening of strains. WT, wild-type. The PCR fragments were further analyzed by DNA sequence determination.

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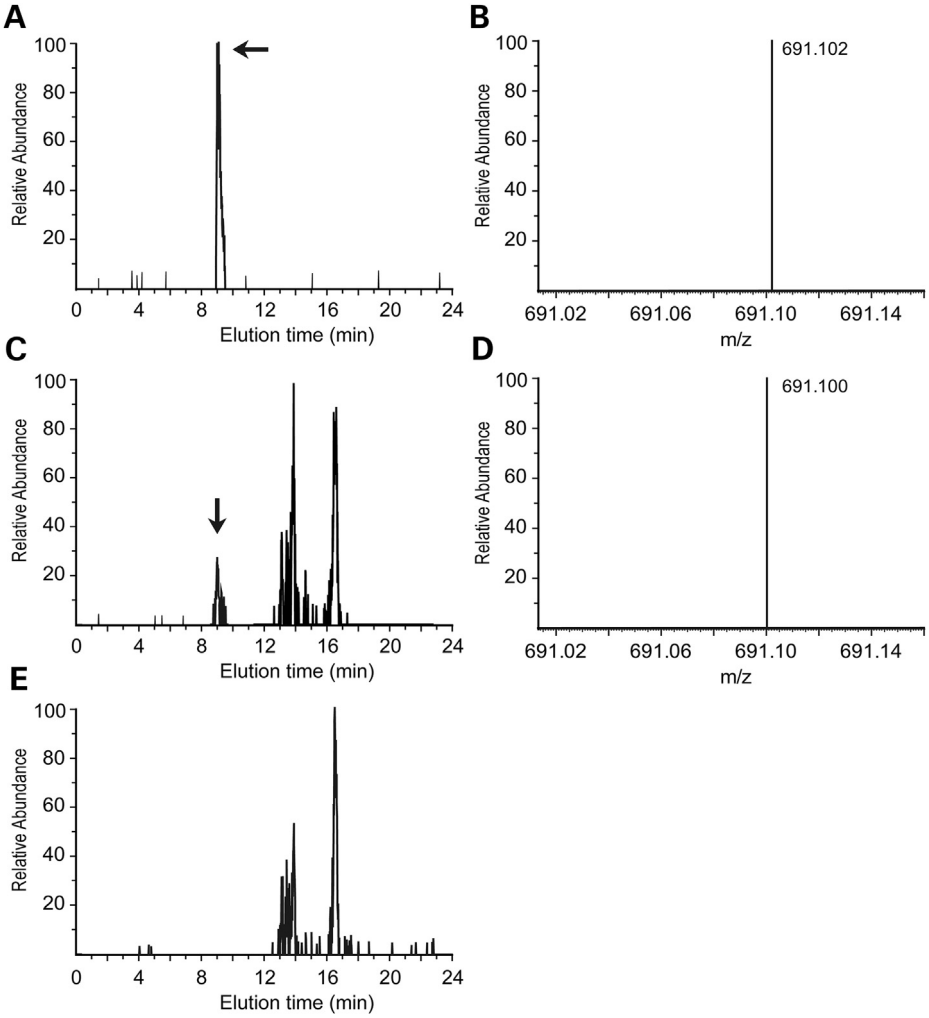

Figure 2. Isolation and determination of intracellular c-di-GMP for anaerobic cultures of wild-type H37Rv and dgc^{mut}. Bacteria were grown in the RAD model and were collected at the onset of anaerobiosis, which was indicated by the decolorisation of methylene blue. After extraction, nucleotides in samples were separated and determined by using LC-MS. The elution peaks at ~9 min (indicated by arrows) for pure c-di-GMP (A) and the wild-type sample (C) were confirmed by mass spectrum (B, D). No c-di-GMP was detected from the dgc^{mut} sample (E).

3.5. *dgc and pde are not required for aerobic growth and long-term survival*

To further test whether c-di-GMP signaling is unnecessary for aerobic growth, as suggested by the transcriptome analyses, we measured growth and survival for the 5 strains. Viable counts of aerobic cultures showed that the growth curves of dgc^{mut}, Δ*pde*, and their repaired/complemented strains were comparable to that of wild-type H37Rv (Figure S1A). After 80 days, the viability of the 5 strains had no obvious difference (Figure S1B). Thus, c-di-GMP signaling is not involved in *M. tuberculosis* viability under aerobiosis.

3.6. *C-di-GMP modulates oxygen consumption*

To determine the timing of oxygen depletion in the RAD model, the oxygen-sensitive dye methylene blue was added to cultures at the time of inoculation. Methylene blue begins to fade as the dissolved oxygen concentration falls below 3% [7], and it decolorizes completely upon anaerobiosis when stage NRP-2 (non-replicating persistence 2) is reached [3]. The blue color in the Δ*pde* culture disappeared after bacterial growth for 162 h, which was comparable to that seen with wild-type cultures (159 h) (Figure 3B). In contrast, the total color-fading time for dgc^{mut} was only 136 h. For dgc^{rep} and pde^{com}, the decolorization times were 163 h and 171 h, respectively. CFU counts were similar for the strains, which excluded the possibility that the differences in oxygen consumption were due to different viable cell numbers. Thus, the *dgc* mutant consumes oxygen more rapidly than the *pde* mutant and the wild-type H37Rv strain, suggesting that c-di-GMP signaling in *M. tuberculosis* mediates oxygen consumption.

3.7. *C-di-GMP modulates anaerobic survival*

Wild-type H37Rv, both mutants, and the repaired/complemented strains grew at similar rates in the RAD model until growth arrest due to oxygen consumption. The viability of dgc^{mut} was slightly higher than that of the wild-type during the entire anaerobic period (Figure 3A). In contrast, Δ*pde* lost viability much faster than the wild-type. By days 20 and 120, Δ*pde* recovery was only 58% and 7.6% of wild-type levels, respectively, indicating a defect in anaerobic survival associated with Δ*pde*. In a different RAD model, the *dosR* mutant also lost viability rapidly, with only 0.01% of wild-type viability being recovered between days 100–200 [7], which was several hundred times faster than seen with Δ*pde*. dgc^{rep} and pde^{com} partially corrected the dormancy defects due to gene deficiencies, but they did not restore wild-type viability

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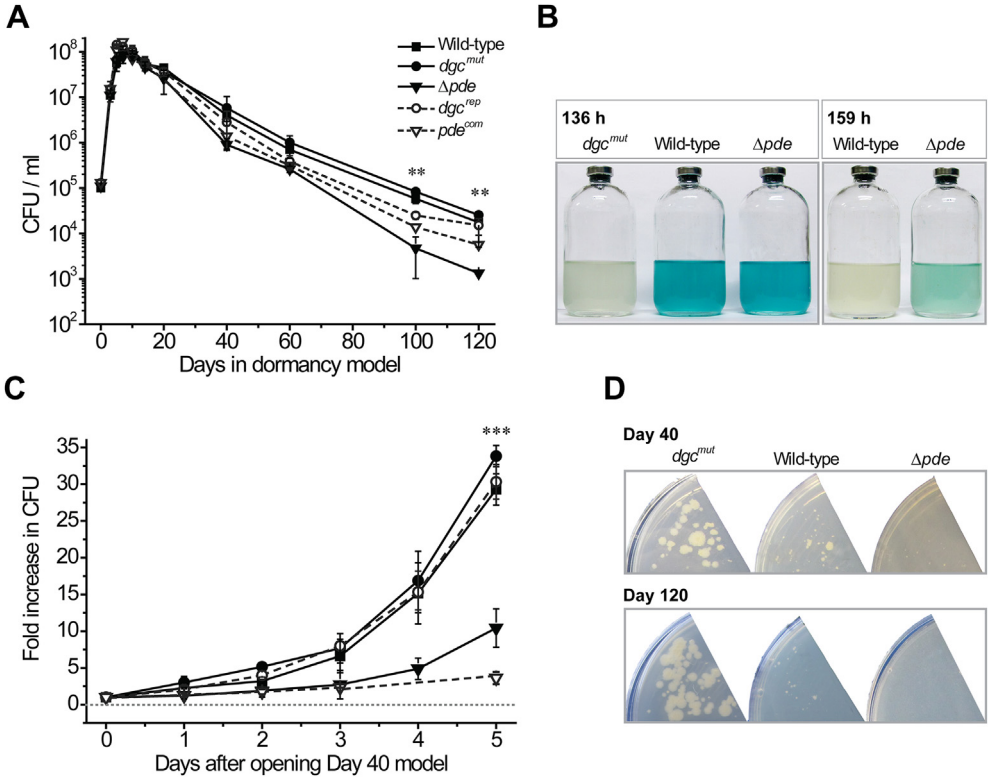

Figure 3. C-di-GMP signaling modulates anaerobic dormancy. (A) Growth and survival during anaerobic dormancy. The wild-type, mutant and complemented bacteria were grown in standard 18-ml serum bottles with a culture-to-headspace ratio of 0.65. Cultures were incubated on a shaker at 240 rpm. Bacterial viability was determined by recovery on solid medium. Data presented here is from one representative experiment with three independent cultures. Each data point was the average of data from three independent cultures. **, $p < 0.004$ (for wild-type H37Rv, *dgc*^{mut} and Δ*pde*). (B) Oxygen consumption (methylene blue decolourization) in cultures of the RAD model. Methylene blue at a final concentration of 4.5 μg/ml was added to cultures during inoculation. One bottle of three independent cultures for each strain was randomly selected for imaging. (C) Growth rate of cultures during recovery from anaerobic dormancy. The day-40 cultures in the model were exposed to oxygen and fresh 7H9 medium at a dilution rate of 1:1, and allowed to resume aerobic growth. Bacterial growth was monitored every day for 5 days by plating on 7H10 agar. The growth rate was calculated by determining the fold increase in the number of CFU in comparison to the CFUs present immediately after the anaerobic cultures were opened, following the methods as described previously [7]. Each data point was the average of data from three independent original anaerobic cultures. ***, $p < 0.001$ (Day 5). (D) Colony formation during recovery from anaerobic dormancy. The anaerobic cultures in the RAD model were diluted and plated onto solid 7H10 agar for CFU counting. Photos of colony growth were taken at the same time point for the same batch cultures.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Figure 3A) possibly due to the elevated burden of the complementing plasmid.

3.8. C-di-GMP modulates recovery from dormancy

When survival of bacteria exposed to the RAD model was measured for more than 10 d, we noticed that wild-type H37Rv took a week longer than *dgc*^{mut} to form colonies on solid agar (Figure 3D). In contrast, colony formation of Δ*pde* lagged about 1-week behind the wild-type strain, which is similar to results obtained with a *dosR* mutant [7]. As the incubation time under anaerobic dormancy lengthened, the time lag for colony formation increased (for the day-120 cultures, colonies of Δ*pde* appeared 1.5 and 3 weeks later in comparison to the wild-type and *dgc*^{mut} bacilli, respectively). However, no difference in colony growth was observed for the 5 strains when they were grown aerobically or were in the early aerobic stages (<6 days) in the RAD model (data not shown). These observations suggest that Δ*pde* is defective in recovery from dormancy.

We next measured the ability of the strains to resume growth upon reintroduction of oxygen to the day-40 anaerobic cultures. After exposure to oxygen and fresh medium, wild-type H37Rv and *dgc*^{mut} began to replicate within one day (Figure 3C). *dgc*^{mut} grew slightly faster than wild-type H37Rv. In contrast, the Δ*pde* mutant took longer to resume growth, with ~30% of the wild-type recovery level on day 5. *pde*^{com} did not recover the wild-type regrowth phenotype. The recovery defect of *pde*^{com} may be due to the elevated burden of the complementing plasmid, as wild-type H37Rv carrying the plasmid was also slow to regrow after exposure to long-term hypoxia/anaerobiosis (data not shown). These observations indicate that c-di-GMP controlled by *dgc* and *pde* modulates *M. tuberculosis* recovery from anaerobic dormancy.

3.9. C-di-GMP modulates survival under a hypoxic and reductive stress

Isoniazide (INH) specifically kills multiplying *M. tuberculosis*; it is not bactericidal with non-replicating, dormant *M. tuberculosis* [3,4]. When treated with 4 μg/ml INH for 1-week, the wild-type, mutant and repaired/complemented strains showed no difference in survival (data not shown). A transient dormancy triggered by the NO donator SNP at 100 mM also showed no difference in survival for the 5 strains (data not shown). Thus, c-di-GMP is unrelated to survival of active bacteria during treatment with INH.

We next asked whether c-di-GMP affects *M. tuberculosis* viability in an *in vitro* hypoxic and reductive condition. Vitamin C, an antioxidant involved in a number of vital cellular functions in humans, rapidly (within a few hours) triggers *M. tuberculosis* growth arrest by scavenging O_2 [6]; a much longer time is needed for hypoxia development in the Wayne model. In the present study, bacterial growth was arrested in the presence of 5 mM vitamin C. The vitamin C-triggered growth arrest promoted bacterial tolerance to INH, with

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survival increasing by 60% by day 4 as compared to the group treated with only INH. In the presence of vitamin C, a subpopulation of bacteria (27–39%) remained susceptible to INH (Figure 4A), suggesting that this subpopulation was active in hypoxic and reductive conditions. After treatment for 7 days with vitamin C and INH, Δ*pde* showed a survival (0.042% of the original viability) that was much lower than for dgc^{mut} (1%) (Figure 4A and B). dgc^{rep} and pde^{com} displayed the wild-type survival level (about 0.2%). Furthermore, after vitamin C treatment, colony formation was faster for dgc^{mut} than for wild-type cells and slower for Δ*pde* by about 1-week (data not shown). These data demonstrated that c-di-GMP modulates *M. tuberculosis* viability to hypoxic and reductive stresses.

3.10. C-di-GMP controls M. tuberculosis growth in THP-1 cells

The enhanced and attenuated *in vitro* survival phenotypes of dgc^{mut} and Δ*pde*, respectively, prompted an examination of the relevance of c-di-GMP to *M. tuberculosis* virulence. The macrophage-like cells of the human cell line THP-1 were infected with bacteria. dgc^{mut} and dgc^{rep} grew at the same rate as wild-type H37Rv (Figure 5A). However, Δ*pde* was nearly arrested in growth, although its intracellular multiplication was not impaired within the first day after post-infection. pde^{com} restored the wild-type growth rate.

Human and murine macrophages (such as THP-1) have the ability to positively transport vitamin C into cells, with intracellular concentrations being over 10 mM [34–36]. Vitamin C was thus used to further assess the intracellular viabilities of dgc^{mut} and Δ*pde*. Addition of 2 mM vitamin C into cultures did not differentiate the intracellular viabilities of dgc^{mut} and wild-type H37Rv

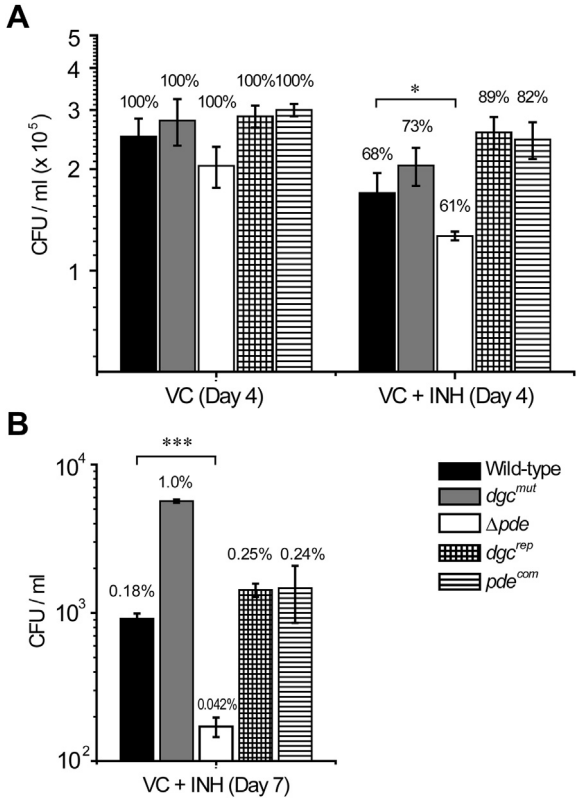

Figure 4. C-di-GMP signaling modulates bacterial survival under a hypoxic and chemotherapeutic condition. (A, B) Bacteria were inoculated into fresh medium to ~10^6 CFU/ml and immediately treated with 5 mM vitamin C (VC). After treatment for 24 h, INH was added to a final concentration of 4 μg/ml. The survivals for the INH-treated cultures were calculated with their corresponding non-INH-treated cultures at day 4. Each data point was the average of data from three independent cultures. *, $p < 0.02$; ***, $p < 0.001$.

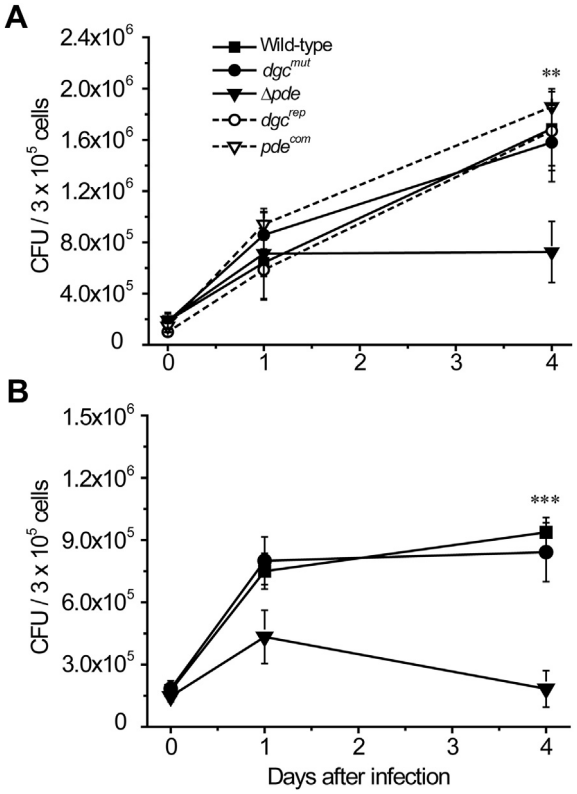

Figure 5. C-di-GMP signaling controls *M. tuberculosis* virulence in human THP-1 cells. MOI 1:1. (A) Intracellular replication within macrophages. Each data point was the average of data of six independent cultures from two independent experiments. **, $p < 0.003$ (Day 4). (B) Bacteriostasis within vitamin C (VC)-treated macrophages. The THP-1 cells were treated with 2 mM vitamin C immediately after bacterial infection. ***, $p < 0.001$ (Day 4).

(Figure 5B). In contrast, the viability of Δ*pde* was reduced to 20% of the wild-type level. We also observed a slow colony-forming phenotype for Δ*pde*(data not shown). Consequently, *pde* is essential for bacterial growth in macrophage-like cells.

3.11. C-di-GMP modulates virulence in mouse models

The attenuated virulence of Δ*pde* in macrophage infection prompted us to investigate whether Δ*pde* also decreases the pathogenicity of host tissues. C57BL/6 mice were infected with wild-type H37Rv or Δ*pde* via tail vein injections. Though an almost identical amount of bacteria was implanted into C57BL/6 mice, the effective loads (day 0) of Δ*pde* in the lungs and spleens were lower than with wild-type H37Rv (Figure 6A and B). The difference in CFUs between the mutant and wild-type strains narrowed between weeks 3 and 6. After infection for 10 weeks, few Δ*pde* bacilli isolated from spleens and lungs were recovered on plates (even after cultivation for 2 months), but wild-type H37Rv maintained viability well (~5×10^6 CFU per gram of tissue). Histopathological analyses showed that lungs infected with the Δ*pde* mutant had more normal tissue than those infected with wild-type H37Rv (Figure 6C). These data support the hypothesis that *pde* (low intracellular c-di-GMP levels) is required for the adaptive survival of *M. tuberculosis* upon entering into the mouse host.

4. Discussion

Several studies have provided clues that *M. tuberculosis* is armed with mechanisms, including but not limited to the DosR regulon, for adaptation to and survival during dormancy [2,6,24]. The data

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described above demonstrate that the intracellular second messenger c-di-GMP is involved in *M. tuberculosis* dormancy, independent of the DosR system (Figure 7). The signaling is positively- and negatively-regulated by the diguanylate cyclase (Dgc) and phosphodiesterase (Pde), as enhanced and attenuated phenotypes were observed in dgc^{mut} and Δ*pde* mutants, respectively.

The c-di-GMP signal transduction pathway in *M. tuberculosis* correlates with the bacterial response to hypoxia. Mutation of the single-copy *dgc* in *M. tuberculosis* eliminates intracellular c-di-GMP. In *M. smegmatis*, mutation of *dgc* also results in an absence of c-di-GMP [28]. Under aerobiosis, the transcriptome profiles of dgc^{mut} and wild-type H37Rv showed no difference. Correspondingly, the same phenotypes were observed for the two strains in both long-term survival and growth rate of aerobic cultures. However, a transcriptome difference was observed between the two strains for anaerobic cultures sampled at the NRP-2 stage, a time at which the color of methylene blue had disappeared [3]. In accordance with the transcription data, dgc^{mut} and Δ*pde* mutants showed opposite phenotypes when compared to wild-type H37Rv in the *in vitro* RAD model.

C-di-GMP in *M. tuberculosis* is involved in bacterial survival and recovery from dormancy. Several *in vitro* models have been developed to investigate *M. tuberculosis* dormancy by mimicking intracellular conditions possibly encountered by this bacterium, including stress caused by hypoxia [3,37], nutrient-limitation, reactive oxygen species, reactive nitrogen species, and acidic conditions [4,6,7,10,12,38]. In our modified Wayne model, the *dgc* mutation slightly enhanced anaerobic survival and the recovery from dormancy, whereas the deletion of *pde* resulted in defects for both phenotypes. Similar to Δ*pde*, both a LuxR family gene mutant we generated (Δ*Rv0195*) [32] and a *dosR* mutant [7] are also defective for the two phenotypes. Vitamin C, an essential nutrient exerting multiple physiological effects in humans, can accumulate to over 10 mM in human cells [34–36]. Vitamin C promotes *M. tuberculosis* dormancy by exerting reductive stress and by creating hypoxia through rapid O_2 scavenging [6]. In the vitamin C-triggered dormancy model, dgc^{mut} showed higher survival than wild-type H37Rv. In contrast, the viability of Δ*pde* was lower than the wild-type, a result that was similar to the lower viability of Δ*Rv0195* [32] and Δ*dosR* [6] in the presence of vitamin C. These data demonstrate a role for c-di-GMP signaling in *M. tuberculosis* on the negative modulation in bacterial hypoxic/anaerobic dormancy.

The dormancy modulation of c-di-GMP signaling does not overlap with the DosR regulon. The DosR system controls the expression of approximately 48 genes upon exposure to hypoxia/NO/CO and other cues [2,9,10,12]. A substantial up-regulation of the DosR regulon was observed in both transcriptomes derived from the anaerobic cultures of dgc^{mut} and wild-type H37Rv compared with their aerobic transcriptomes (Table S3). However, no difference was observed in the transcription profiles of the DosR regulon genes between dgc^{mut} and the wild-type stain under either aerobic or anaerobic conditions. The result suggests that *dosR* and c-di-GMP modulates *M. tuberculosis* dormancy by a different signaling pathway. However, the c-di-GMP signaling pathway possibly interacts with the *Rv0195*-mediating network to regulate *M. tuberculosis* dormancy, as 18 genes displayed opposite transcriptional patterns in dgc^{mut} and Δ*Rv0195* [32].

We propose that c-di-GMP modulates *M. tuberculosis* anaerobic dormancy by mediating a metabolic mechanism and survival-related proteins. Mutation of *dgc* causes *M. tuberculosis* to consume oxygen faster than wild-type H37Rv. Similarly, the *dosR* mutant consumes oxygen more rapidly than the wild-type [7]. However, the absence of *dgc* and *dosR* produced opposite physiological effects on bacterial anaerobic survival, suggesting that they mediate different pathways. Most of the down-regulated genes in the anaerobic dgc^{mut} cultures are involved in metabolism, in addition to several ribosomal proteins and the principal sigma factor, SigA, but not 12 other sigma factors responsible for stress response [39]. Upon exposure to hypoxia, the down-regulation of SigA may provide dgc^{mut} with a dormancy-

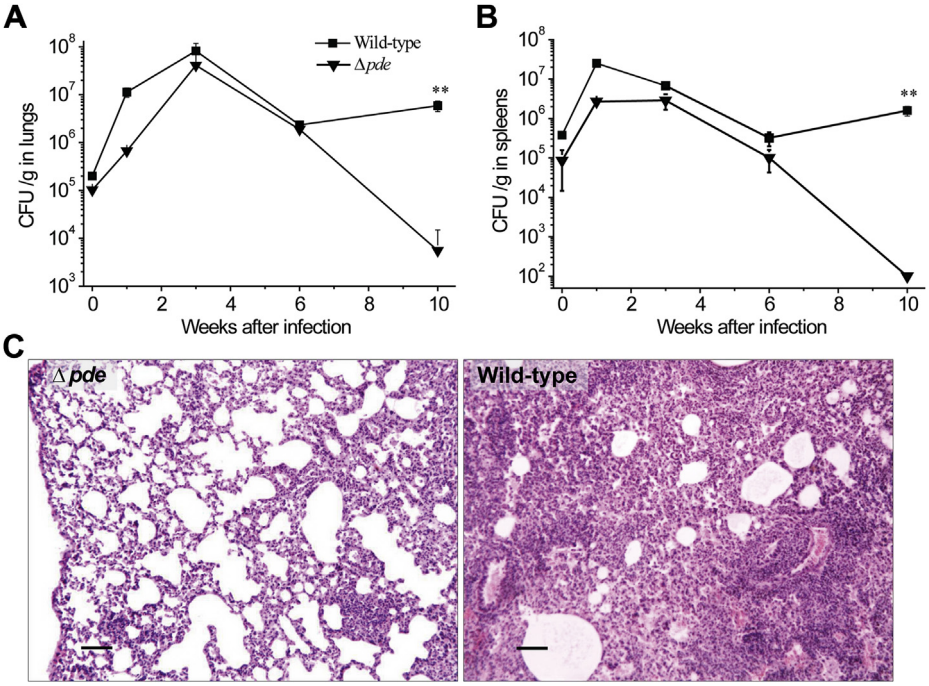

Figure 6. C-di-GMP signaling controls *M. tuberculosis* pathogenicity in mouse infection. Bacteria of $\sim 10^6$ cells were implanted per mouse via tail vein injection. Δ*pde* did not multiply as well in the lungs (A) or the spleens (B) as wild-type H37Rv. After 3 w of infection, the bacilli of Δ*pde* isolated from organs showed slower colony formation than the wild-type. Each data point was the average of data from three to four independent mice. **, $p < 0.004$ (Week 10). (C) Histopathology of lungs of C57BL/6 mice. At 6 w, the lesions were large and dense in wildtype-infected mouse lungs compared to the lesions in Δ*pde*-infected mouse lungs, in which more normal lung tissue was preserved. Magnification, ×100; Scale bar, 100 μm.

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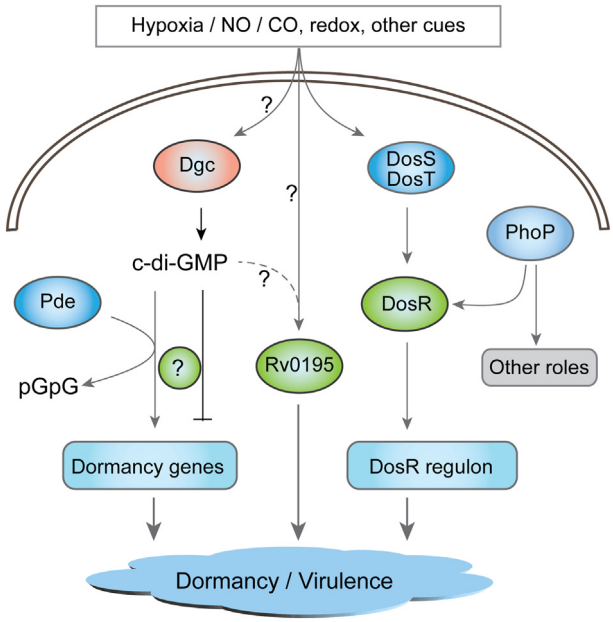

Figure 7. Model of *M. tuberculosis* dormancy. Firstly, under hypoxia or other conditions, DosT directly senses decrease of O_2 while DosS senses a redox change, via the heam group inside their N-terminal GAF domains, activating the downstream transcription regulator DosR. DosT and DosS are also activated by CO and NO [16–20]. Activation of DosR results in expression of the DosR regulon [6,9,10], thus enhancing bacterial dormancy [2,6,7]. DosR is also partially regulated by PhoP [22]. Secondly, Rv0195, a LuxR family regulator, modulates bacterial dormancy by a mechanism independent on the DosR regulon [32]. Thirdly, in the c-di-GMP signaling pathway, Dgc senses hypoxia or other cues by an unknown mechanism and mediates synthesis of the second messenger c-di-GMP. High levels of c-di-GMP decrease *M. tuberculosis* dormancy via (an) unknown receptor(s) responsible for the expression of dormancy-related genes. Pde breaks down c-di-GMP, thus promoting bacterial dormancy. The c-di-GMP signaling pathway partially overlays with the Rv0195-leading dormancy modulation [32] but not with the DosR regulon. The pivotal genes mediating dormancy also participate in bacterial virulence.

promoting mechanism to prepare the bacteria for adaptation to and survival from a more severe hypoxia. Interestingly, several genes were up-regulated in dgc^{mut}, including *eis*, which enhances the intracellular survival of *M. tuberculosis* in human cells [30,33]. These molecular events explain the enhanced and attenuated dormancy of dgc^{mut} and Δ*pde*, respectively.

It is likely that *M. tuberculosis* inside mammalian hosts experiences repeated alternation between dormancy and reactivation and that the key genes mediating dormancy participate in bacterial virulence (Figure 7). The dormancy-weakened Δ*pde* mutant showed defects in virulence during infection of the human THP-1 macrophage-like cells. The dormancy-weakened Δ*pde* mutant showed defects in virulence during infection of the human THP-1 macrophage-like cells and C57BL/6 mice. Similar inhibitory effects of c-di-GMP on virulence exist for a number of other pathogens [40] including *Vibrio cholerae* [41], *Salmonella* Typhimurium [42] and *Pseudomonas aeruginosa* [43]. Further evidence for the relevance of dormancy genes with *M. tuberculosis* virulence concerns *dosR* and *Rv0195*. Both *dosR* and *Rv0195* [32] mutants showed significant defects not only in hypoxic/anaerobic dormancy survival [2,7], but also in cell infection models [6] and animal infection models, including guinea pigs and rabbits [23]. However, the role of *dosR* on virulence in mice is controversial [2,23,44]. It should be noted that mouse models do not produce hypoxia severe enough to trigger *M. tuberculosis* dormancy [45]. Subtle variations in experimental methods might result in the slightly different results for *dosR* mutants, since the *in vivo* growth and dormancy of *M. tuberculosis* depend on the balance between host immunity and bacterial virulence. We observed that the Δ*pde* mutant developed a dormancy-like phenotype in mice, as the bacilli isolated from mouse lungs and spleens exhibited delays in colony formation. Therefore, it appears that mutations of key dormancy regulators weaken bacterial pathogenicity with hosts that trigger *M. tuberculosis* dormancy, but they may not decrease bacterial growth in hosts lacking an ability to trigger bacterial dormancy. Dormancy regulators, such as *pde*, *Rv0195* [32] and *dosR* [7,46], do not alter bacterial growth kinetics under aerobic conditions.

C-di-GMP is reported to be required for survival of several microbes including *M. smegmatis* [28,47]. Both *M. tuberculosis* and *M. smegmatis* harbor a conserved Dgc with GAF, GGDEF, and EAL domains. *M. tuberculosis* contains an additional EAL protein (Pde), and *M. smegmatis* contains another GGDEF protein. In *M. smegmatis*, deletion of *dgc* did not affect growth [28], which is consistent with our observations in *M. tuberculosis*. *M. smegmatis* lacking *dgc* exhibited defects in aerobic long-term survival under nutritional starvation [28]. However, we found that the absence of *dgc* in *M. tuberculosis* did not affect aerobic long-term survival, but enhanced anaerobic survival. In contrast, the absence of *pde*, which probably increased intracellular c-di-GMP at particular times, reduced anaerobic survival. It remains to be determined how *dgc* and *pde* regulate intracellular c-di-GMP levels to control *M. tuberculosis* behaviors. The opposite results obtained for *M. tuberculosis* and *M. smegmatis* suggest that c-di-GMP plays different roles in these pathogenic and non-pathogenic mycobacterial species.

In conclusion, our work provided a new insight into the mechanisms of *M. tuberculosis* dormancy, and extended the knowledge of the physiological roles mediated by c-di-GMP in bacteria. Our preliminary work with the avirulent *M. tuberculosis* H37Ra indicated that chemically-synthesized c-di-GMP restores the dgc^{mut} transcriptome to that of the Δ*pde* mutant (unpublished data), suggesting that c-di-GMP can penetrate into mycobacterial cells and act as a signaling molecule. C-di-GMP is an effective immunomodulator, and it might influence the host response to bacterial pathogens [48,49]. Our findings demonstrate that c-di-GMP negatively regulates *M. tuberculosis* dormancy and virulence. C-di-GMP or its analogues may thus be a candidate for enhancing the control of latently-infected tuberculosis and shortening clinical chemotherapeutic regimens.

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Competing interests: None declared.

Ethical approval: All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guideline of Beijing Tuberculosis and Thoracic Tumour Research Institute (Permit Number: M101230-1). All efforts were made to minimize animal number and suffering.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2013.09.002.

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