

Mycobacterium bovis BCG Response Regulator Essential for Hypoxic Dormancy

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Received 26 June 2002/Accepted 23 September 2002

Obligately aerobic tubercle bacilli are capable of adapting to survive hypoxia by developing into a nonreplicating or dormant form. Dormant bacilli maintain viability for extended periods. Furthermore, they are resistant to antimycobacterials, and hence, dormancy might play a role in the persistence of tuberculosis infection despite prolonged chemotherapy. Previously, we have grown dormant *Mycobacterium bovis* BCG in an oxygen-limited Wayne culture system and subjected the bacilli to proteome analysis. This work revealed the upregulation of the response regulator Rv3133c and three other polypeptides (α -crystallin and two “conserved hypothetical” proteins) upon entry into dormancy. Here, we replaced the coding sequence of the response regulator with a kanamycin resistance cassette and demonstrated that the loss-of-function mutant died after oxygen starvation-induced termination of growth. Thus, the disruption of this dormancy-induced transcription factor resulted in loss of the ability of BCG to adapt to survival of hypoxia. Two-dimensional gel electrophoresis of protein extracts from the gene-disrupted strain showed that the genetic loss of the response regulator caused loss of the induction of the other three dormancy proteins. Thus, the upregulation of these dormancy proteins requires the response regulator. Based on these two functions, dormancy survival and regulation, we named the Rv3133c gene *dosR* for dormancy survival regulator. Our results provide conclusive evidence that DosR is a key regulator in the oxygen starvation-induced mycobacterial dormancy response.

Mycobacteria are obligate aerobes, i.e., they require oxygen for growth. However, ample evidence from animal models and human studies suggests that tubercle bacilli encounter hypoxic environments in active disease as well as in latent infection (30, 32, 33, 38, 41, 42). L. G. Wayne established a link between starvation for oxygen and drug resistance. He demonstrated that upon gradual depletion of oxygen from a culture (oxygen-limited Wayne culture system), the bacillus exits the cell cycle and develops into a defined dormant form that is adapted to hypoxia and maintains viability for extended periods of time. Importantly, the dormant form of the bacterium is resistant to antimycobacterials. This phenotypic resistance could be due to the fact that antimycobacterials target mainly growth-related functions such as cell wall synthesis (15, 34–40). Hence, an emerging working model suggests that phenotypically drug-resistant dormant bacilli, similar to the ones observed in oxygen-starved cultures, exist in hypoxic microenvironments in the host and may cause persistent infection despite prolonged chemotherapy; currently, 6 to 9 months of treatment is required to cure the patient of tuberculosis (4, 8, 9, 18, 21, 24).

Obviously, this monocausal model of persistence oversimplifies the situation in vivo. However, if it does reflect some aspects of the life of this parasite in its host, the development of drugs that target hypoxic dormant bacilli could have a profound impact on tuberculosis therapy. A combination therapy consisting of conventional antimycobacterials (targeting growing bacilli) and antidormancy drugs (hitting the dormant sub-

population) might considerably shorten the time necessary for treatment (6, 41).

Mycobacterium tuberculosis is an aerosol-transmitted bio-safety level 3 pathogen. To overcome the associated experimental limitations, we established the attenuated BCG strain of *M. bovis* as a model organism to study the hypoxic life of the tubercle bacillus. We demonstrated that BCG shows a dormancy response that is strikingly similar to the behavior shown by *M. tuberculosis* when grown in the oxygen-limited Wayne culture system (11, 12, 16, 20, 22, 29). To identify dormancy-induced proteins, we previously subjected BCG grown in the Wayne system to a proteome analysis (1). In this work, the response regulator Rv3133c, a putative phosphorylation-dependent transcription factor identified in the *M. tuberculosis* H37Rv genome project (2), was found to be upregulated immediately upon entry into dormancy. In contrast, this protein is not detectable in either aerated growing or aerated stationary-phase cultures. The temporal expression profile in the Wayne system and the apparent dormancy specificity, together with the probable biochemical function of the protein as a transcription factor, suggest that the molecule could play a key regulatory role in the adaptation of bacilli to survival under hypoxic conditions (1).

Together with the response regulator, three other proteins, α -crystallin (5, 14, 17, 26, 31, 36, 43, 44) and two “conserved hypothetical” proteins, universal stress protein domain-containing Rv2623 (7, 14, 19, 26, 31) and cystathionine β -synthase domain-containing Rv2626c (14, 26, 31) are upregulated upon entry into dormancy (1). The observed temporal coinduction of these three proteins along with the response regulator upon entry into dormancy could indicate that they are under the control of this transcription factor. To determine whether Rv3133c plays a role in adaptation to hypoxic survival and in

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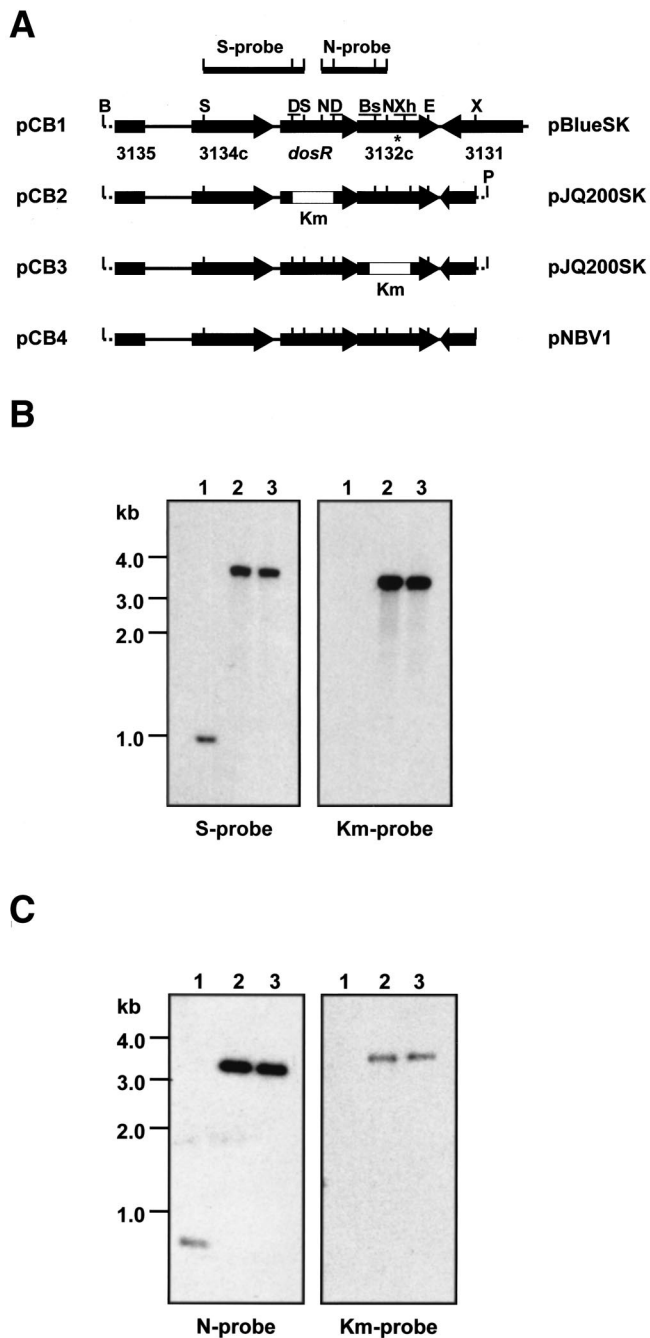


FIG. 1. *dosR* locus, gene replacement constructs, rescue plasmid, and Southern blot analyses of gene replacement mutants. (A) Schematic diagrams (not to scale) of the *dosR* locus in BCG (pCB1) and derivatives thereof (pCB2 to -4) are shown. pCB2, *dosR* gene replacement plasmid; pCB3, *Rv3132c* gene replacement plasmid; pCB4, rescue plasmid. Horizontal arrows show open reading frames, and the numbers below the arrows in pCB1 indicate the corresponding *Rv* numbers of open reading frames as annotated for the *M. tuberculosis* H37Rv genome (2). Empty boxes labeled Km represent the kanamycin resistance cassette. Only relevant restriction sites are indicated: B, *Bam*HI; S, *Sph*I; D, *Dra*III; N, *Not*I; Bs, *Bst*XI; Xh, *Xho*I; E, *Eco*RI; X, *Xba*I; P, *Pst*I. Underlined frames as annotated for deletion of coding sequences. S-probe and N-probe indicate *Sph*I and *Not*I fragments used as probes for the Southern blots shown in B and C, respectively. Solid line, genomic DNA; dotted line, polylinker sequence. Vector backbones of the plasmids are indicated on the right

the upregulation of the three coinduced proteins, we deleted its coding sequence from the BCG genome. We report the phenotypic consequences of this loss-of-function mutation on growth and viability and the proteome of the bacillus grown in the Wayne dormancy culture system.

MATERIALS AND METHODS

Strain, media, cultivation, and monitoring of growth and survival. All experiments were conducted with *Mycobacterium bovis* BCG Pasteur strain ATCC 35734 at 37°C. Liquid medium was Dubos Tween-albumin broth; solid medium was Dubos oleic-albumin agar (Difco). Kanamycin, gentamicin, and hygromycin were from Sigma. Standard (i.e., oxygen-unlimited) cultures (100 ml) were grown in plastic roller bottles (10 by 14 cm) at 1 rpm and with a starting *A*₆₀₀ of 0.05. The containers were opened daily to allow exchange of air (1). Oxygen-limited Wayne cultures were grown in screw-cap test tubes (20 mm by 125 mm), with a total volume of 25.5 ml. Precultures were diluted to an *A*₆₀₀ of 0.005 in a final volume of 17 ml. Magnetic stirrers were added; the cultures were sealed by tightly screwing down solid caps with latex liners and stirred gently at 170 rpm on magnetic stirring platforms (1, 16, 39).

Oxygen depletion was monitored via decolorization of the redox indicator methylene blue as described previously (16). Growth and survival were determined by turbidity measurements in a Bacharach Coleman model 35 photometer (Thomas Scientific) and by determination of CFU on agar after plating of appropriate dilutions as described previously (16). In contrast to the hypoxic stationary-phase cells grown in the Wayne system, clumping of bacilli occurred throughout aerated stationary-phase growth in roller bottles. To reduce clump-

side. pBlueSK, pBluescriptSK (Stratagene); pJQ200SK, suicide vector (25); pNBV1, *E. coli*-mycobacterium shuttle vector (10). The asterisk indicates the site of a single-base-pair polymorphism that was detected between BCG and *M. tuberculosis* H37Rv. Codon 283 of *Rv3132c* in H37Rv (ATT) is ACT in BCG, resulting in an Ile-to-Thr amino acid replacement in the sensor domain of the kinase. (B) Southern blot analyses of $\Delta dosR::km$ gene replacement mutants. S-probe, DNA from the wild type (lane 1) and two independently isolated *dosR* gene replacement mutants (lanes 2 and 3) was digested with *Sph*I and *Eco*RI, transferred to a membrane, and probed with the 1-kb *Sph*I fragment (S-probe) shown in A. BCG wild-type genomic DNA showed the expected hybridization band, corresponding to the size of the *Sph*I fragment used as the probe. In contrast, the $\Delta dosR::km$ mutants, which have lost an *Sph*I site due to deletion of the *dosR* coding sequence, showed a band of 3.7 kb, corresponding to the expected size of the genomic *Sph*I-*Eco*RI fragment containing the disrupted $\Delta dosR::km$ allele (compare pCB1 with pCB2). Km-probe, probing of the blot with a 1.2-kb *Pst*I fragment carrying the kanamycin resistance cassette detected the same hybridization band in the $\Delta dosR::km$ strains that was detected by using the 1-kb *Sph*I fragment as the probe, confirming that the 3.7-kb *Sph*I-*Eco*RI band observed for these strains indeed contained the kanamycin resistance cassette and, hence, the disrupted *dosR* gene. (C) Southern blot analyses of $\Delta Rv3132c::km$ gene replacement mutants. N-probe, DNA from the wild type (lane 1) and two independently isolated *Rv3132c* gene replacement mutants (lanes 2 and 3) was digested with *Not*I and *Xba*I, transferred to a membrane, and probed with the 0.8-kb *Not*I fragment (N-probe) shown in A. Wild-type genomic DNA showed the expected hybridization band, corresponding to the size of the *Not*I fragment used as the probe. In contrast, the $\Delta Rv3132c::km$ mutants, which have lost a *Not*I site due to deletion of the *Rv3132c* coding sequence, showed a band of 3.2 kb corresponding to the expected size of the genomic *Not*I-*Xba*I fragment containing the disrupted $\Delta Rv3132c::km$ allele (compare pCB1 and pCB3). Km-probe, probing of the blot with the 1.2-kb *Pst*I fragment carrying the kanamycin resistance cassette (Km-probe) detected the same hybridization band in the $\Delta Rv3132c::km$ strains that was detected with the 0.8-kb *Not*I fragment as a probe, confirming that the 3.2-kb *Not*I-*Xba*I band in BCG $\Delta Rv3132c::km$ genomic DNA contained the kanamycin resistance cassette and hence the disrupted gene. DNA from both the $\Delta dosR::km$ and $\Delta Rv3132c::km$ mutants showed no hybridization signal with the pJK200SK vector as the probe, confirming that the strains had lost all vector sequences (data not shown). Size markers are from the 1-kb ladder; 1 to 2 μ g of genomic DNAs was loaded.

ing for CFU determinations, the roller bottle cultures were sonicated five times for 15 s each with 1-min ice breaks at level 2 of an Ultrasonic processor XC sonicator (Misonix) before plating.

DNA manipulations. DNA manipulations were done according to standard protocols (28). DNA purifications were carried out with Qiagen kits as recommended by the supplier. DNA was introduced into BCG by electroporation as described before (29) with minor modifications. A lambda ZAPII library of genomic DNA from BCG strain ATTC 35734 was constructed with Stratagene reagents (B. Murugasu-Oei and T. Dick, unpublished data). A 429-bp probe specific to *dosR* was generated by PCR with the primers TCGTAGGTGAG-GCGGGTTC and CGGCGATCTGCTTGTGGT, designed according to the sequence of the gene in *M. tuberculosis* H37Rv (Rv3133c [2]). The library was screened, and positive clones were excised as pBluescriptSK plasmids with helper phages according to the protocol provided by the supplier.

One clone (pCB1, Fig. 1A) carrying a 5.9-kb insert containing the *dosR* gene plus flanking regions was subjected to DNA sequencing on a Perkin-Elmer ABI Prism 377 sequencer. To facilitate subsequent manipulations of the *dosR* operon, a 4.7-kb *Bam*HI-*Xba*I fragment (*Bam*HI site in the polylinker of pBluescriptSK, *Xba*I site downstream of Rv3132c within the genomic fragment) was excised from pCB1 and ligated with *Bam*HI- and *Xba*I-digested pNEB193 (New England Biolabs). The resulting plasmid was digested with *Dra*III, resulting in a 607-bp deletion within the 654-bp *dosR* coding sequence. The product was blunt ended with T4 DNA polymerase and ligated with the blunt-ended, 1.2-kb *Pst*I fragment carrying the kanamycin resistance gene from pUCK4 (Pharmacia). The 5.4-kb genomic DNA fragment carrying the kanamycin resistance cassette replacing the *dosR* coding sequence was released via *Bam*HI-*Pst*I digestion (*Pst*I site in the polylinker of pNEB193) and ligated into *Bam*HI- and *Pst*I-digested pJQ200SK (25), resulting in the gene replacement plasmid pCB2 (Fig. 1A).

pNEB193 carrying the 4.7-kb *Bam*HI-*Xba*I fragment was also used to generate the replacement construct for the Rv3132c coding sequence. The plasmid was digested with *Bst*XI and *Xho*I, resulting in an 895-bp deletion within the 1,737-bp Rv3132c coding sequence. The product was blunt ended and ligated with the kanamycin resistance cassette as before. The 5.1-kb genomic DNA fragment carrying the kanamycin resistance cassette replacing the Rv3132c coding sequence was transferred to pJQ200SK as before, resulting in the Rv3132c gene replacement plasmid pCB3 (Fig. 1A). Sequencing the deletion sites showed that the kanamycin resistance cassette was inserted in the same transcriptional orientation as *dosR* and Rv3132c. Gene replacement was carried out according to the two-step protocol, taking advantage of the properties of the suicide vector pJQ200SK to confer sensitivity to sucrose and resistance to gentamicin (23).

Briefly, BCG was transformed with 1 μ g of the gene replacement constructs and selected on agar containing kanamycin (20 μ g ml⁻¹). Replica plating of kanamycin-resistant colonies on agar containing sucrose (2%) and gentamicin (5 μ g ml⁻¹) identified strains that were sucrose sensitive and gentamicin resistant, indicating genomic recombination of the whole-gene replacement constructs. These recombinants were grown in broth to allow a second, intrachromosomal recombination step to occur. For selection of second-step recombinants that had lost the pJQ200SK sequence and the flanking copy of the native, wild-type genes, cultures were plated on agar containing sucrose and kanamycin. Sucrose- and kanamycin-resistant colonies were replica plated on agar containing gentamicin to confirm gentamicin sensitivity and thus loss of the pJQ200SK sequence. For rescue of the phenotypes caused by disruption of *dosR*, the 4.7-kb *Bam*HI-*Xba*I genomic fragment containing the *dosR* operon was released from pNEB193 (see above) via *Bam*HI and *Xba*I digestion and ligated into *Bam*HI- and *Xba*I-digested pNBV1, which confers resistance to hygromycin (50 μ g ml⁻¹) (10), resulting in pCB4 (Fig. 1A).

Two-dimensional gel electrophoretic analysis. Bacilli were broken up by disrupting the cells with glass beads in a Mini Beadbeater cell disruptor as described previously (1). Then 100 μ g of total protein was subjected to isoelectric focusing with pH 4 to 7 Immobililine dry strips and an IPGphor isoelectric focusing unit (Amersham Pharmacia) and separated in the second dimension by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (Protean IIx system; Bio-Rad) as described before (1).

Nucleotide sequence accession number. The sequence of the *dosR* locus as shown in pCB1 (Fig. 1A) has been deposited in GenBank under accession number AF522461.

RESULTS

Generation of BCG Δ *dosR*::*km*. The *dosR* (Rv3133c) locus was isolated from a BCG genomic library and sequenced. Figure 1A (pCB1) shows the structural organization of the *dosR*

region in BCG, which was found to be identical to that of *M. tuberculosis* H37Rv (2). At 30 bp upstream of the *dosR* coding sequence is the 3' end of the Rv3134c open reading frame (conserved hypothetical protein). Overlapping the TGA stop codon of *dosR* is the ATG start codon (italic) of Rv3132c (CCA TGA) encoding a sensor histidine kinase (27). This genomic organization suggests that the three genes form an operon. In vivo promoter probing confirmed the presence of dormancy-inducible promoter activity in the intergenic region upstream of Rv3134c and the absence of such activity upstream of *dosR* and Rv3132c (B. H. Tan, C. Boon, and T. Dick, unpublished observations). This proposed operon structure is consistent with recent transcriptional studies of the locus (3, 31).

To determine the function of DosR, its coding sequence was replaced with a kanamycin resistance cassette (Fig. 1A, pCB2; see Materials and Methods for details). The replacement of the *dosR* gene by its inactivated allele (Δ *dosR*::*km*) was confirmed by Southern blotting, as shown in Fig. 1B.

Hypoxia-induced death of BCG Δ *dosR*::*km*. To determine the role of the *dosR* gene in the dormancy response, BCG Δ *dosR*::*km* was grown in the oxygen-limited Wayne culture system. This system is based on growth of the bacilli in sealed tubes with stirring (16, 39). Initially the cultures grow exponentially and consume oxygen. A temporal oxygen gradient is self-generated, and upon encountering a microaerobic threshold level after about 4 to 5 days, the wild type is deflected from exponential growth and enters a transition phase in which the turbidity of the culture increases slowly up to day 10, after which the A_{600} stays constant (Fig. 2A) (1). Wild-type bacilli in the hypoxic stationary phase are in a state of dormancy, i.e., they are nonreplicating and maintain viability (16, 39).

In the initial aerobic exponential growth phase observed in this system, BCG Δ *dosR*::*km* was indistinguishable from the wild type (Fig. 2A). This shows that the loss of *dosR* did not affect the growth of the bacillus. BCG Δ *dosR*::*km* terminated aerobic exponential growth at the same time (i.e., after 4 to 5 days) and at the same A_{600} as the wild type. However, in contrast to the wild type, BCG Δ *dosR*::*km* underwent the transition from exponential growth to constant turbidity by day 6, i.e., the characteristic extended transition phase of the wild type has been lost in the Δ *dosR*::*km* strain. This result could indicate that *dosR* is involved in the transition (adaptation) from aerobic exponential growth to hypoxic nonreplicating survival.

To determine the effect of the *dosR* loss-of-function mutation on the actual viability of the hypoxic nongrowing culture, CFU were determined. Figure 2B shows that the CFU of BCG Δ *dosR*::*km* and the wild type were identical up to day 5 in the Wayne system, when both strains terminated aerobic exponential growth. Consistent with the slight increase in A_{600} over the next 5 days during the extended transition phase, a moderate increase in CFU was observed for the wild type at day 10. After that, the CFU of the wild type stayed constant for 40 days of total cultivation time, after which the experiments were terminated. In contrast to the survival of hypoxia shown by the wild type, Fig. 2B shows that the CFU of BCG Δ *dosR*::*km* started to decline at day 10. After 40 days of total incubation time, the viable-cell count of the BCG Δ *dosR*::*km* culture was 1,500-fold lower than that at day 5, when exponential growth terminated (Fig. 2B).

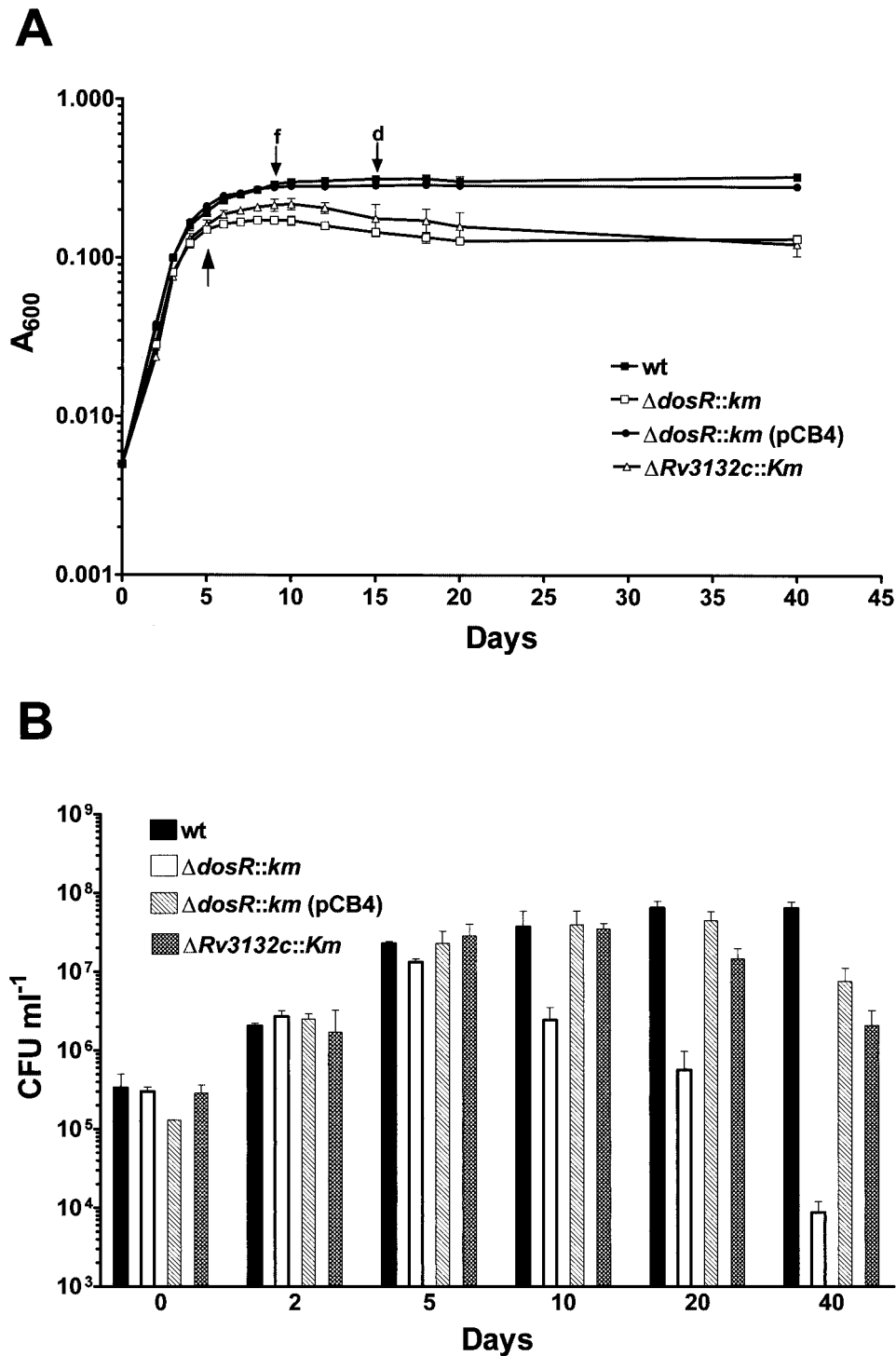


FIG. 2. Growth and survival of wild-type BCG and $\Delta dosR::km$, $\Delta dosR::km$ (pCB4), and $\Delta Rv3132c::km$ strains in the Wayne dormancy culture system. Exponentially growing cultures were diluted and grown in sealed tubes with stirring. (A) Growth monitored by turbidity measurement. (B) Growth monitored by colony count determination. wt, wild type; $\Delta dosR::km$, *dosR* gene replacement mutant; $\Delta dosR::km$ (pCB4), $\Delta dosR::km$ mutant transformed with the *dosR* rescue plasmid pCB4; $\Delta Rv3132c::Km$, *Rv3132c* gene replacement mutant (see Fig. 1A). Growth and survival of $\Delta dosR::km$ transformed with pNBV1, i.e., the vector backbone used to carry the *dosR* genomic fragment in the pCB4 rescue construct (Fig. 1A), was indistinguishable from the behavior of the untransformed $\Delta dosR::km$ strain (data not shown). The experiments were carried out three times with duplicate cultures. Standard deviations are shown. The arrow pointing upwards indicates the time at which samples were taken for the protein analyses shown in Fig. 3. f and d, fading and complete decolorization of the oxygen indicator methylene blue, respectively.

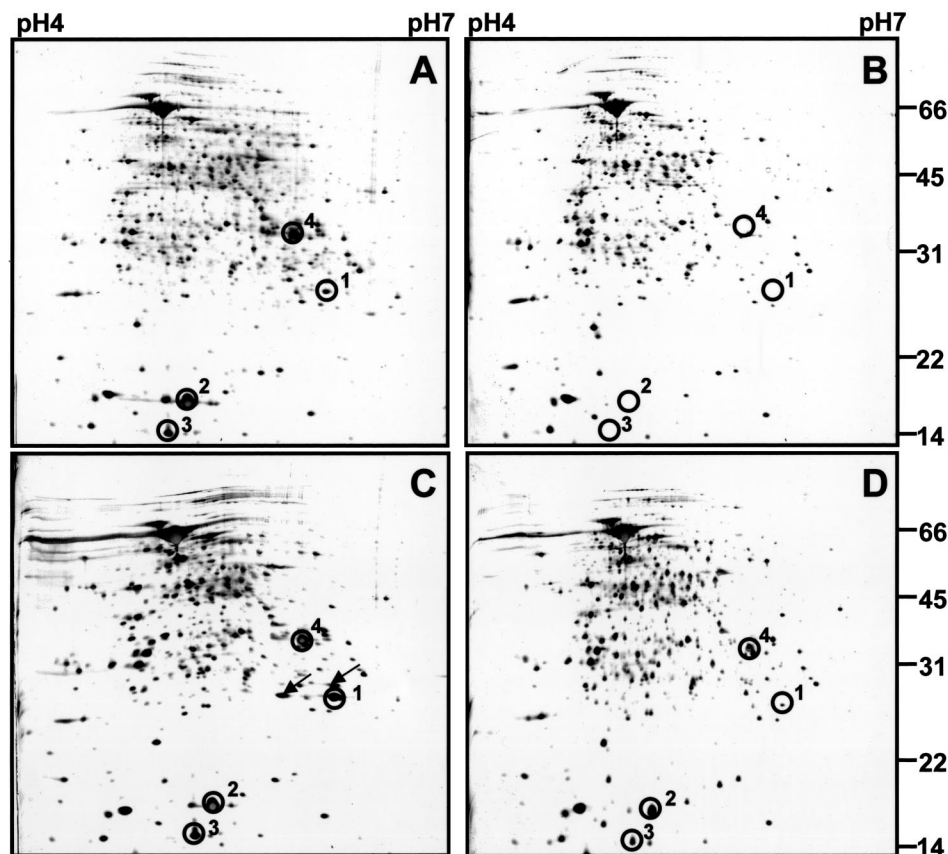


FIG. 3. Two-dimensional gel electrophoretic analyses of protein extracts from wild-type BCG and $\Delta dosR::km$, $\Delta dosR::km(pCB4)$, and $\Delta Rv3132c::km$ strains grown in the Wayne dormancy culture system. Protein extracts were prepared at the time when cultures grown in the Wayne system terminated growth at day 5, as indicated by the arrow in Fig. 2A. Then 100- μ g samples of total protein were subjected to two-dimensional gel electrophoresis. Silver-stained gels are shown. (A) Wild-type BCG. (B) BCG $\Delta dosR::km$. (C) BCG $\Delta dosR::km(pCB4)$. Two protein spots that were detected in the pCB4-transformed $\Delta dosR::km$ strain upon termination of growth are marked by arrows; their identity remains to be determined. (D) BCG $\Delta Rv3132c::km$. Circles labeled 1 to 4 indicate dormancy-induced protein spots. In B, where the dormancy proteins were not detectable, the circles indicate their expected migration positions. 1, DosR; 2, α -crystallin; 3, conserved hypothetical protein Rv2626c; 4, conserved hypothetical protein Rv2623. Sizes are indicated in kilodaltons. The experiments were repeated twice with independently prepared cultures, yielding the same results. The experiments were also carried out for all four strains with protein extracts from 20-day-old Wayne cultures, yielding the same results that were obtained for 5-day-old cultures (data not shown).

The growth and survival analyses were carried out with an independently isolated $\Delta dosR::km$ strain (Fig. 1B, lane 3), yielding identical results (data not shown). Figure 2 also shows that both phenotypes exhibited by BCG $\Delta dosR::km$, loss of the extended transition phase and hypoxic death, were rescued by a wild-type copy of the *dosR* operon carried by plasmid pCB4 (Fig. 1A). Taken together, these results show that the *dosR* disruption affected the transition phase and caused massive death of the oxygen-starved culture. Hence, *dosR* is essential for the adaptation of BCG to survival of hypoxia.

Loss of dormancy proteins in BCG $\Delta dosR::km$. Previously we demonstrated that DosR is upregulated immediately upon oxygen starvation-induced termination of growth and that α -crystallin and the two conserved hypothetical proteins Rv2623 and Rv2626c are temporally coinduced with the response regulator (Fig. 3A) (1). To reveal a possible regulatory role of the *dosR* gene in the induction of these dormancy proteins, BCG $\Delta dosR::km$ was grown in the Wayne culture system, and a proteome analysis was carried out. Protein ex-

tracts were prepared from cultures that were terminating growth (Fig. 2A, arrow) and were subjected to two-dimensional gel electrophoresis. BCG $\Delta dosR::km$ had lost the DosR protein, as expected (compare protein spot numbered 1 in Fig. 3A with empty circle numbered 1 in Fig. 3B).

Strikingly, Fig. 3B also shows the loss of induction of all three dormancy proteins that were coinduced with DosR in wild-type bacilli. The proteome analysis was carried out with the independently isolated $\Delta dosR::km$ strain (Fig. 1B, lane 3), yielding identical results (data not shown). Figure 3C shows that the regulatory phenotype shown by BCG $\Delta dosR::km$ could be rescued by a wild-type copy of the *dosR* operon carried by plasmid pCB4 (Fig. 1A). These results demonstrate that the induction of α -crystallin, Rv2623, and Rv2626c by dormancy depends on DosR and thus that DosR plays a regulatory role in the dormancy response.

Minor phenotypic consequences in BCG $\Delta Rv3132c::km$. The apparent cotranscription of the histidine kinase Rv3132c (27) with the *dosR* response regulator (see Fig. 1A, pCB1) could

indicate that the two proteins form a two-component system in which the kinase is involved in phosphorylation of DosR. Thus, Rv3132c could play the role of a dormancy sensor kinase, regulating DosR activity. To determine whether the kinase is indeed essential for the dormancy response, the gene was replaced with a kanamycin resistance cassette (Fig. 1A, pCB3; Fig. 1C), and the resulting $\Delta Rv3132c::km$ strain was grown in the Wayne culture system.

Figure 2A shows that the $\Delta Rv3132c::km$ strain appeared to maintain a wild-type-like extended transition phase, although the turbidity of the culture at day 10 was somewhat lower. Viability of the hypoxic, nongrowing BCG $\Delta Rv3132c::km$ culture showed a moderate 15-fold reduction after 40 days of growth in the Wayne system, compared to the 1,500-fold hypoxic kill observed for the $\Delta dosR::km$ mutant under the same conditions. Consistent with the moderate effect of the loss of kinase function on growth and survival in the Wayne culture system, Fig. 3D shows that BCG $\Delta Rv3132c::km$ maintained inducible expression of the dormancy proteins; although the induced level of the dormancy proteins appeared to be somewhat reduced compared to the induction of the proteins in the wild type. The growth, survival, and proteome analyses were carried out with an independently isolated $\Delta Rv3132c::km$ strain (Fig. 1C, lane 3), yielding identical results (data not shown).

BCG $\Delta Rv3132c::km$ thus showed a moderate phenotype compared to the loss of extended transition phase, loss of hypoxic viability, and loss of expression of dormancy proteins observed for BCG $\Delta dosR::km$ when grown in the Wayne culture system. These results indicate that, in comparison to the key role observed for *dosR*, the Rv3132c kinase plays a minor role in the dormancy response. Furthermore, these results confirm that the phenotypic effects of the *dosR* inactivation were not due to polar effects on the downstream-located Rv3132c kinase gene.

Wild-type-like survival of BCG $\Delta dosR::km$ in aerated stationary phase. Previously we demonstrated that upregulation of DosR is specific to oxygen starvation-induced termination of growth, i.e., to the dormancy response. Termination of growth by nutrient starvation under non-oxygen-limited conditions in aerated roller bottles is not accompanied by an increase in the steady-state level of DosR (1). Based on the lack of DosR induction in aerated stationary-phase cultures, one would predict that the protein does not play a role in the survival of aerated nongrowing culture. To test the dormancy specificity of *dosR* function, BCG $\Delta dosR::km$ was grown in aerated roller bottles, and growth and survival were determined. Both strains terminated exponential growth after 5 days of cultivation, at an A_{600} of 0.8. They showed an identical transition phase and the same viable-cell counts in the aerated stationary phase after a total cultivation time of 20 days (8×10^7 and 6×10^7 CFU ml⁻¹ for the wild-type and BCG $\Delta dosR::km$ strains, respectively) and 40 days (3×10^6 and 2×10^6 CFU ml⁻¹ for the wild-type and BCG $\Delta dosR::km$ strains, respectively). These results show that growth and survival of BCG $\Delta dosR::km$ under non-oxygen-limited conditions in roller bottles were indistinguishable from those of the wild type. Hence, the function of *dosR* in maintaining the viability of nongrowing cultures is specific for hypoxic dormancy, and dormancy-specific induc-

tion of DosR correlates with the dormancy specificity of its function.

DISCUSSION

Oxygen starvation triggers an adaptive dormancy response in the obligately aerobic tubercle bacillus. Survival of hypoxia and upregulation of the response regulator DosR (Rv3133c), α -crystallin, and the conserved hypothetical proteins Rv2623 and Rv2626c are hallmarks of the dormant organism (1). We show here that DosR is a key regulator of this response. Disruption of the *dosR* gene resulted in a more than 1,000-fold loss of viability in oxygen-starved nongrowing culture. This shows that *dosR* function is essential for adaptation to hypoxic survival. At the proteome level, we demonstrated that the genetic loss of *dosR* resulted in loss of induction of the other dormancy-induced proteins, indicating that expression of these proteins is under the control of DosR. Whether the dependence of expression of the dormancy proteins on DosR is direct (via activation of the promoters of the dormancy proteins) or indirect remains to be elucidated.

The deletion of the coding sequence of the histidine kinase Rv3132c located downstream of *dosR* had moderate consequences on hypoxic survival and induction of dormancy proteins. This suggests that the Rv3132c kinase plays only a minor role in the dormancy response and that other histidine kinases could be involved in dormancy signaling. The situation in the signal transduction pathway in *Mycobacterium* dormancy could thus be similar to sporulation signaling in *Bacillus subtilis*, in which multiple histidine kinases control (via a complex phosphorelay system) the phosphorylation status of the master regulator of sporulation, the response regulator Spo0A (13).

Our finding that α -crystallin induction depends on *dosR* but not on the Rv3132c histidine kinase is consistent with data from Sherman et al. (31). Those authors were unable to generate a *dosR* gene replacement mutant (as a "result of technical difficulties?" [31]). Hence, they interfered with the expression of *dosR* by interrupting the conserved hypothetical protein gene Rv3134c, which is located upstream of *dosR* (see Fig. 1A). By employing an anti- α -crystallin antibody, it was shown that this mutation resulted in loss of induction of α -crystallin under low-oxygen culture conditions. Complementation experiments suggested that this was due to reduced *dosR* expression and not to the inactivation of Rv3134c (31). In contrast, an Rv3132c histidine kinase-disrupted strain maintained inducible α -crystallin expression. Consistent with our data, the Rv3134c-disrupted strain did not show significant loss of viability in stationary-phase cultures grown in aerated roller bottles. Growth and survival of the Rv3134c and Rv3132c mutant strains in an oxygen-limited, Wayne-type culture system were not characterized by the authors (31).

The identification of DosR as an essential dormancy response regulator in our present study promises rapid progress in understanding the biochemistry underlying the quiescent hypoxic life of these obligate aerobes. The $\Delta dosR::km$ strain allows definition of the complete regulon (and thus the dormancy-essential enzymatic functions) under control of this transcription factor by comparative DNA microarray analyses. Even more important, perhaps, BCG $\Delta dosR::km$ represents the first *Mycobacterium* mutant that shows not only a strong but

also an apparently specific survival phenotype in hypoxic dormant bacilli generated in the Wayne culture system. We demonstrated previously that DosR is not detectable in aerated stationary-phase culture (1), and we show here that, consistent with that observation, survival of the *dosR* loss-of-function mutant was indistinguishable from that of the wild type under these conditions. Thus, the Δ *dosR::km* mutant provides us with a probe for testing the relevance of hypoxic dormant bacilli for the life of the parasite in vivo.

If hypoxic dormant bacilli do play a role in the persistence of the bacilli during drug treatment, one would predict that chemotherapy of active disease and/or latency caused by the Δ *dosR::km* strain is more effective than the treatment of wild-type infection. DosR loss-of-function bacilli cannot weather the onslaught of conventional drugs by "hiding" in their dormant form, and hence, the time required to eradicate infection should be reduced. Furthermore, hypoxic dormancy of the bacillus might play a role in the progression of active disease and/or in the establishment and maintenance of latency of infection. If that is the case, the Δ *dosR::km* mutant is expected to show attenuated virulence. Animal models are in place to test these predictions (8). Thus, DosR and its dormancy regulation could provide a rich source of targets for a fundamentally new level of chemotherapeutic intervention against tuberculosis. Not hitting growth-related targets in proliferating bacilli but knocking out dormancy-related targets in sleepers may turn out to be the way to go in the development of future, more effective antimycobacterials.

ACKNOWLEDGMENTS

We thank Alice Tay, Genome Analysis Laboratory, IMCB, for help with DNA sequencing and Indrajit Sinha and Michael Box for comments on the manuscript. We thank Bill Bishai for pNBV1 and M. F. Hynes for pJQ200SK.

T.D. is an adjunct staff member of the Department of Microbiology, National University of Singapore. This work was supported by the Agency for Science, Technology and Research (A*STAR), Singapore.

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