

Deletion of the *Mycobacterium tuberculosis* α -Crystallin-Like *hspX* Gene Causes Increased Bacterial Growth In Vivo

Yanmin Hu,¹ Farahnaz Movahedzadeh,² Neil G. Stoker,² and Anthony R. M. Coates^{1*}

Medical Microbiology, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, London SW17 0RE,¹ and Department of Pathology and Infectious Diseases, The Royal Veterinary College, London NW1 0UT,² United Kingdom

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Hypervirulent mutants of *Mycobacterium tuberculosis*, whose growth rates are higher in vivo, have now been reported to have mutations in both regulatory and structural genes, but the basis for this unusual phenotype is not understood. One hypervirulence gene, *dosR* (*devR*, Rv2031c), activates transcription of approximately 50 genes in this pathogen in response to hypoxia and nitric oxide stress. The most dramatic activation (~80-fold) is activation of the *hspX* (*acr*, Rv2031c) gene, which encodes a 16-kDa α -crystallin-like protein that is a major antigen. In this study we found that a Δ *acr* mutant exhibited increased growth following infection of BALB/c mice in vivo and in both resting and activated macrophages in vitro (as measured by the number of CFU). The increased growth in macrophages was equal to that of a Δ *dosR* mutant, while introduction of a constitutively expressed *hspX* gene reduced the Δ *dosR* virulence to wild-type levels. These results suggest that the increased number of CFU of the Δ *dosR* mutant was largely due to loss of *hspX* expression. We also confirmed that constitutive expression of *hspX* slows growth in vitro, and we propose that *hspX* plays an active role in slowing the growth of *M. tuberculosis* in vivo immediately following infection.

Much of the success of *Mycobacterium tuberculosis* as a pathogen and our failure to control this microorganism are due to the ability of the organism to survive in an apparently latent state for long periods within caseous or closed lesions (50). Thus, one-third of the world's population is believed to be latently infected, and in 5 to 10% of individuals bacilli will reactivate and cause active disease at some point during their lives (or each year when there is human immunodeficiency virus-*M. tuberculosis* coinfection) (9, 35). The underlying mechanisms by which *M. tuberculosis* enters a persistent state, retains its viability during latency, and reactivates remain largely unknown. Understanding these mechanisms should open up new avenues for improving the control and treatment of tuberculosis in the future.

One factor that has been repeatedly reported to be associated with experimental models of persistence is hypoxia (52), suggesting that hypoxia may be a key signal for inducing latency in vivo. Therefore, we expected that clarification of the bacterium's response to low oxygen concentrations should yield insights into the mycobacterial latent state. The hypoxic response in *M. tuberculosis* is controlled by the *dosRS* two-component regulatory system (2CS) (43) and involves induction of transcription of approximately 50 genes; the predicted functions of these genes include adaptation to hypoxia (e.g., nitrate reductase gene *narX*) and protection from nitrogen stress (e.g., nitroreductase genes *acg*, Rv3127, and Rv3131), but most functions remain unknown and therefore the genes can provide no obvious clues as to how latency is initiated.

The most dramatic transcriptional induction during hypoxia

(~80-fold; *dosR* dependent) is the induction of the *hspX* gene (*acr*, Rv2031c), which encodes a 16-kDa α -crystallin-like protein. This protein, originally defined by using the monoclonal antibody TB68 (4), is an immunodominant antigen that is recognized by the majority of patients with active tuberculosis (2, 15, 20). It is a member of the α -crystallin-like heat shock protein (α -HSP) (6) family and acts in at least some circumstances as a molecular chaperone, preventing thermally induced aggregation of other proteins (3, 53). How this function could contribute to the hypoxic or latency response is not clear, but the protein has been reported to be present in a thickened cell wall in the late stationary phase (5).

An *M. tuberculosis* mutant in which *hspX* was replaced by a hygromycin resistance gene was previously shown to be attenuated in a macrophage model (54). In contrast, deletion of *dosR* (the gene that controls *hspX*) has been reported to cause hypervirulence in mouse models and in activated macrophages (31), although a mutant was attenuated in guinea pigs (22). In order to reconcile the different phenotypes of the *dosR* and *hspX* mutants, we generated an unmarked *hspX* deletion mutant of *M. tuberculosis*. We found marked phenotypic differences compared to the previous report and higher numbers of CFU in mice and macrophages.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* XL1 was used as a host strain for cloning and plasmid propagation. *M. tuberculosis* H37Rv was used as a parental strain to construct the *hspX* mutant. TAME16, a Δ *dosR* H37Rv mutant, has been described previously (31). *M. tuberculosis* was grown in 7H9 medium containing 0.05% Tween 80 supplemented with 10% albumin-dextrose complex (ADC) (Difco Laboratories, Detroit, Mich.) without shaking. Viability (as determined by the number of CFU) was estimated initially and at 4- to 10-day intervals. To count CFU, three 100- μ l samples from appropriate serial 10-fold dilutions were plated onto plates of 7H11 agar supplemented with oleic acid-albumin-dextrose complex (Difco). Antibiotics were used at the following con-

* Corresponding author. Mailing address: Medical Microbiology, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, London SW17 0RE, United Kingdom. Phone: 44 (208) 725 5725. Fax: 44 (208) 672 0234. E-mail: acoates@sghms.ac.uk.

centrations: ampicillin (Sigma), 100 µg/ml; kanamycin (Sigma), 20 µg/ml; gentamicin (Sigma), 20 µg/ml; and hygromycin (Life Technologies), 100 µg/ml.

DNA manipulations, PCR, and sequencing. DNA manipulations were performed by using standard procedures (40). PCR was performed in a 50-µl (total volume) mixture containing 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, each primer at a concentration of 1 µM, 10 ng of DNA, 1 U of Hotstart *Taq* polymerase (QIAGEN), and a buffer supplied with the enzyme. PCR amplification was performed for 30 cycles consisting of 94°C for 1 min, 50 to 58°C (depending on the set of primers) for 2 min, and 72°C for 3 min, followed by a final extension of 7 min at 72°C. PCR products were purified from an agarose gel using a QIAquick gel extraction kit (QIAGEN). The sequences of the purified PCR products were determined with a DNA sequencer (ABI377) using the AmpliTaq FS enzyme for cycle sequencing with dRhodamine dye terminators (Cambridge BioScience Ltd., United Kingdom). Both DNA strands were sequenced by using the two primers that were used to generate each PCR product.

***hspX* mutant construction.** Mutant construction was carried out using the pNIL and pGOAL plasmids and a two-step strategy, as described previously (33). The 2,496 bp of the PCR product containing the *hspX* gene and 1-kb flanking sequences adjacent to each end of the gene was amplified using *M. tuberculosis* H37Rv genomic DNA as the template and primers HX1 (5'-AATAAGCTTTCAGCGTGCAGGTGGCAAG-3') and HX2 (5'-AATAAGCTTACGGTGTTCGCCACATCC-3'). The PCR product was cloned into the HindIII site of pGEM3Z (Promega) to obtain pGEMHX. The *hspX* gene was deleted by PCR performed with primers HXM1 (5'-AATCTCGAGAACTGACCACTGGGTCGCTG-3') and HXM2 (5'-AATCTCGAGGGTGGCCATTTGATGCCTCC-3'), which were designed outwardly starting from the start and stop codons of the *hspX* gene using pGEMHX as the template. The PCR product, which contained only the flanking sequences of the *hspX* gene, was cut with XhoI and ligated to obtain pGEMHXM. The *hspX* flanking sequences without the *hspX* gene were cloned into the HindIII site of p2NIL to obtain p2NILHX. Finally, a *hyg-sacB* marker cassette from the pGOAL19 plasmid was cloned into the PacI site of p2NILHX to obtain the final mutant construct, p2NILHX3. p2NILHX3 DNA was electroporated into *M. tuberculosis* H37Rv cells (32), and the mutant was selected as described previously (33).

To complement the *hspX* deletion with the gene transcribed from its own promoter, a 761-bp DNA fragment containing the *hspX* gene and 216 bp of upstream sequence was amplified by PCR using primers HXC1 (5'-AATAAGCTTGTACCATGGTGTCCGGCAT-3') and HXC2 (5'-AATAAGCTTGGCAGTAATTCGCCAGCA-3'). The PCR product was cloned into the HindIII sites of the integrating plasmid pUC-Gm-Int (21). The resulting construct (pUC-Gm-Int-HXC) was electroporated into the *hspX* mutant, and Gm^r transformants were selected.

To complement the *hspX* deletion with the gene transcribed from a constitutive promoter, the *hspX* gene was amplified by PCR using primers hspX-Bam1 (5'-CGCGGATCCAATGGCCACCACCTTCCCGTT-3') and hspX-Bam2 (5'-CGAGGATCCTCAGTTGGTGGACCGGATCTGAAT-3'). These primers introduced a BamHI site at the 5' and 3' ends, allowing *hspX* to be cloned in frame into plasmid pEM37 (a gift from Edith Machowski), producing pFM167, which expressed *hspX* from the *Ag85A* promoter. The HindIII cassette from pUC-Gm-Int carrying the *int* gene and *att* site was cloned into the HindIII site of pFM167 to produce pFM171, and the orientation of *hspX* was verified by DNA sequencing.

Protein analysis. Protein extraction from the *M. tuberculosis* was performed as described previously (14).

Estimation of viability under stress conditions. *M. tuberculosis* strains were grown in 7H9 medium containing 0.05% Tween 80 supplemented with 10% ADC for 4 and 30 days. A series of 10-ml standing cultures were used for determination of the number of CFU after exposure to stress. For heat shock the cultures were shifted to 53°C, and the numbers of CFU were determined at 0, 10, 20, 30, 40, 60, 90, and 120 min. For anaerobic stress, cultures with loosened caps were incubated under strictly anaerobic conditions (checked with anaerobic indicators [Oxoid]) in a jar (GasPak 150 system; Becton Dickinson) in which H₂ and CO₂ were generated with GasPaks (Oxoid). For acidic stress, the culture medium was replaced with acidic 7H9 medium (pH 4). For oxidative stress and alcohol shock, H₂O₂ (10 mM and 20 mM) or ethanol (5%) was added to the cultures. For nitric oxide stress, a diethylenetriamine-nitric oxide adduct was added to the cultures at final concentrations of 5, 10, and 15 mM, and the cultures were incubated at 37°C. The numbers of CFU for the treated cultures and the nontreated cultures were determined at 0, 4, 7, 15, and 20 days (anaerobic and acidic conditions) or at 0, 24, and 48 h (H₂O₂, diethylenetriamine-nitric oxide, and alcohol). Each stress treatment was carried out in triplicate.

Mouse infection model. Female BALB/c mice (body weight, 18 to 20 g; Harlan UK Ltd.) were used. *M. tuberculosis* bacilli were resuspended in phosphate-buffered saline and were inoculated intravenously (i.v.) by using 2 × 10⁴ CFU per mouse. At various times, spleens and lungs from three or four mice were removed rapidly after sacrifice and transferred into 2-ml tubes, each containing 1 ml of sterile distilled water and 2-mm-diameter glass beads. The lungs and spleens of the mice were homogenized using a reciprocal shaker (Thermo Hybrid Ltd.) for 40 s at speed 6.5. The homogenates were diluted, and the numbers of CFU were determined. Samples of lungs and spleens from two mice for each time were fixed in 10% buffered formalin. For each sample, four slides were prepared (two slides with Ziehl-Neelsen staining and two slides with hematoxylin and eosin staining) and examined. The mouse experiments were carried out three times. In the second and third experiments, the bacteria were passaged from the first and second experiments, respectively.

Macrophage infection. Bone marrow-derived macrophages were cultured for 7 days as described previously (46). Adherent macrophages were harvested and seeded at a concentration of 2 × 10⁵ cells per well into 24-well plates in culture medium without L-cell conditioned medium, penicillin, and streptomycin. For the macrophage J774A.1 cell line, the cells were grown to 80% confluence and seeded as described above. Activation of macrophage cells was carried out by addition of gamma interferon (IFN-γ) (100 U/ml; R&D Systems) for 24 h, followed by addition of lipopolysaccharides (200 ng/ml; Sigma) for 3 h. The cells were infected with the bacterial strains at a multiplicity of infection of 1:1 for 4 h and then washed six times with warm Hanks balanced salt solution. At zero time and 1, 2, 5, and 7 days after infection, the cells were washed and lysed with 0.1% Triton X-100, and the numbers of CFU were determined. At each time, the infected macrophages in two wells were individually harvested using trypsin-EDTA and stained for acid-fast bacilli in order to check macrophage viability. The experiments were carried out three times in triplicate.

RNA extraction and semiquantitative RT-PCR. For extraction of mouse RNA, the right lungs of BALB/c mice were removed from the mice and immediately fixed with RNAlater RNA stabilization reagent (QIAGEN) and then kept at -20°C. Thirty milligrams of the fixed lungs was used for RNA extraction by using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (QIAGEN) to remove contaminating genomic DNA. The sequences of the oligonucleotides used for the cytokines and β-actin were as follows: for interleukin-6 (IL-6), 5'-AGGAGACTCACA GAGGAT-3' (sense) and 5'-TCATGTACTCCAGGTAGCT-3' (antisense); for IL-10, 5'-CTGCCTGCTCTTACTGAC-3' (sense) and 5'-CCTTGATTTCTGG GCCAT-3' (antisense); for tumor necrosis factor alpha (TNF-α), 5'-CACGCT CTTCTGTCTACTG-3' (sense) and 5'-TTGAAGAGAACCTGGGAGT-3' (antisense); for IFN-γ, 5'-GCTACACACTGCATCTTG-3' (sense) and 5'-CTGTT GCTGAAGAAGGTAG-3' (antisense); for IL-12p40 5'-GTAGAGGTGGACT GGACT-3' (sense) and 5'-TGGTGCTTCACACTTCAG-3' (antisense); and for β-actin, 5'-ATGGATGACGATATCGCT-3' (sense) and 5'-ATGAGGTAGTC TGTCAGGT-3' (antisense). Total RNA (1 µg) was used to synthesize cDNA. The RNA was transcribed in a 20-µl (total volume) mixture containing 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 2.5 µM antisense primer, 5 mM dithiothreitol, 40 U of RNasin (Promega), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 200 U of SuperscriptII (Life Technologies). The reverse transcription (RT) reaction was carried out at 42°C for 1 h. Ten microliters of diluted cDNA was used for PCR amplification with the gene-specific primers described above. Each RT-PCR was repeated twice. The PCR products were separated in a 1.2% agarose gel, and this was followed by transfer in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to a Hybond-N+ filter (Amersham). The specific probe for each cytokine was amplified using mouse DNA as the template and the gene-specific primers described above and was labeled with [α -³²P]dCTP (specific activity, >3,000 Ci mmol⁻¹; ICN) by using the random priming method according to the instructions of the manufacturer (Amersham). The blots were hybridized with the specific probes overnight. After washing, the blots were scanned using a STORM 840 phosphorimaging instrument at 50-µm pixel resolution. The image files were analyzed using the ImageQuANT software (Molecular Dynamics). The quantity of each PCR product was standardized by determining the ratio of the intensity of the cytokine PCR band to the intensity of the β-actin band and was expressed as the relative level of mRNA.

RESULTS

Construction of an *M. tuberculosis hspX* deletion mutant.

The *hspX* gene is the first gene in a probable operon consisting of four genes (*hspX*, *Rv2030c*, *pfkB*, and *Rv2028c*) (Fig. 1A).

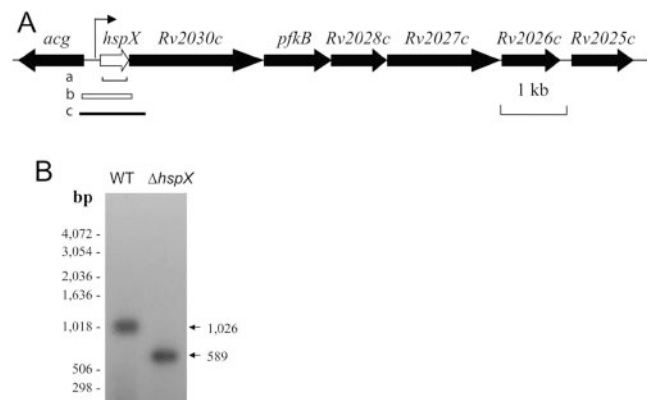


FIG. 1. Construction of an *M. tuberculosis hspX* mutant. (A) Genomic context of *hspX*. The open arrow shows the location of the *hspX* gene which was deleted in the mutant. Bar a indicates the deletion in *hspX*. Open box b shows the location of the *hspX* complement fragment. The bent arrow indicates the transcription start site of the *hspX* gene. Bar c shows the EcoRV fragment used as a probe for Southern blot analysis. (B) Southern hybridization showing the *hspX* deletion. DNA from the WT and $\Delta hspX$ strains were digested with EcoRV and hybridized with the 1,014-bp probe shown in panel A.

All four of these genes have short (0- to 16-bp) intergenic gaps, and all are coincided in microarray studies in a *dosR*-dependent manner (19, 34). It was therefore critical to construct an *hspX* mutation that did not affect expression of the downstream genes. An in-frame deletion of the entire *hspX* gene was constructed in *M. tuberculosis* H37Rv (Fig. 1A), producing strain YH*hspX* Δ 1. PCR (not shown) and Southern blot analysis (Fig. 1B) were used to confirm the presence of the mutation. In order to complement the mutation, a 761-kb DNA fragment containing the *hspX* coding region and 216 bp upstream of it (Fig. 1A) was cloned into an integrating plasmid to generate pUC-Gm-Int-HXC. The construct was transformed into *M. tuberculosis* YH*hspX* Δ 1, producing strain YH*hspX* Δ 1-comp.

Lack of HspX expression in the mutant was demonstrated by analysis of total protein extracted from cultures of the wild-type (WT), YH*hspX* Δ 1, and YH*hspX* Δ 1-comp strains which had been incubated for 4 to 100 days. The cellular levels of the 16-kDa protein were low in log-phase cultures of the WT strain but were high in the stationary-phase cultures, which is consistent with previous findings (12, 53). No visible 16-kDa protein was found in the YH*hspX* Δ 1 cultures, but this protein was found in the complemented strain (data not shown).

Response to stress conditions. Many members of the α -HSP family act as stress response proteins, although expression of the *hspX* gene is not heat inducible (14, 47). We therefore studied the effects of the *hspX* mutation on the bacterium's response to a variety of stress conditions. No significant differences in survival between the WT and the mutant were observed upon treatment with heat (55°C for 2 h), nitric oxide, H₂O₂, acid (pH 4), or anaerobic conditions (data not shown). These results support previously described results which showed that HspX is not induced in response to ethanol, H₂O₂, carbon starvation, cold shock, or resuspension in spent medium (53).

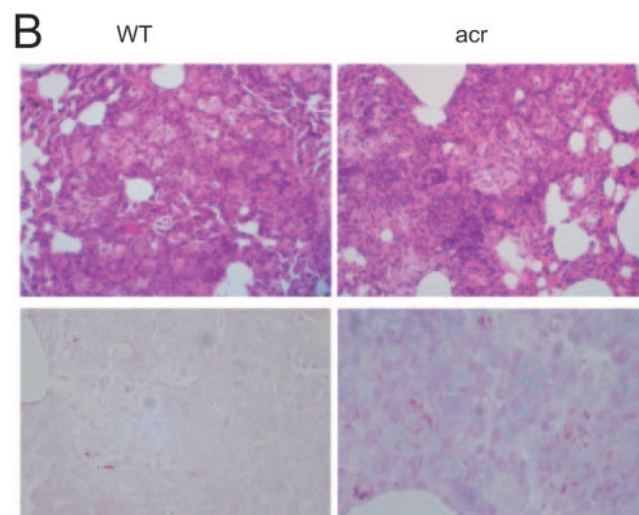
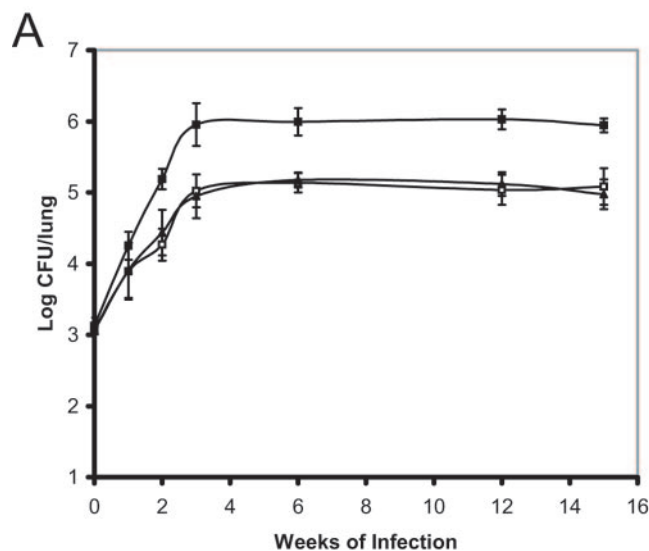


FIG. 2. Growth of *M. tuberculosis* $\Delta hspX$ in vivo. (A) Survival in a BALB/c mouse immune stasis model. Mice were infected i.v. with 2×10^4 *M. tuberculosis* cells, and the numbers of total lung CFU were determined. The values are means derived from three independent experiments in which we used three or four mice at each time. \square , H37Rv (WT); \blacksquare , $\Delta hspX$; \blacktriangle , *hspX*-Comp. The counts for time zero were obtained from the mouse tissues after 2 h of infection. The numbers of CFU of the mutant recovered from the lungs were significantly higher (as determined with a *t* test) than the numbers of CFU of H37Rv at week 1 (1.7-fold; $P < 0.05$), week 2 (9-fold; $P < 0.0001$), and 3 to 15 weeks (10-fold; $P < 0.0001$). (B) Histology of lungs taken from mice infected with H37Rv (WT) and $\Delta hspX$ (*acr*) and stained with hematoxylin and eosin (top panels) (14 weeks after infection; magnification, $\times 20$) and Ziehl-Neelsen stain (bottom panels) (15 weeks after infection; magnification, $\times 40$).

Growth of *M. tuberculosis* $\Delta hspX$ in vivo. A medium-dose i.v. mouse infection model (13, 29) was used to examine the growth and persistence of the mutant. This model is a well-defined model in which there is exponential growth for approximately 3 weeks, followed by a plateau as the acquired immune system controls but fails to eliminate the infection. BALB/c mice were infected i.v. with 2×10^4 CFU of the WT, YH*hspX* Δ 1, or YH*hspX* Δ 1-comp strain. As shown in Fig. 2A, the

number of CFU of the WT strain reached 10^5 CFU/lung 3 weeks following infection and remained constant until week 15. In contrast, YH*hspX* Δ 1 grew significantly more rapidly in the lungs, and the concentration reached 10^6 CFU/lung before plateauing at 3 weeks. The complemented strain YH*hspX* Δ 1-comp behaved like the WT strain, confirming that the phenotype was *hspX* dependent (Fig. 2A). The numbers of CFU in spleens showed a similar pattern (data not shown).

In order to investigate the mechanism of hypervirulence, we carried out a histopathological examination of parts of the lungs and spleens from BALB/c mice infected with the WT and mutant strains. After 14 weeks, the lungs from mice infected with both strains showed large areas of granulomatous inflammation containing lymphoplasmacytic cells, macrophages, neutrophils, and multinucleated cells, with changes consistent with early granuloma formation. Although there was a suggestion that larger areas were involved in inflammation and granuloma formation in the mutant, the analysis did not demonstrate that there were significant differences in tissue damage (Fig. 2B). However, larger numbers of acid-fast bacilli were present in the inflammatory cell foci of lungs from YH*hspX* Δ 1-infected mice (Fig. 2B), which is consistent with the CFU profiles. In addition, spleens from YH*hspX* Δ 1-infected mice were 25 to 40% larger at 2, 3, and 6 weeks ($P = 0.001$) (data not shown), indicating that there was an increased inflammatory response.

In order to determine if the overgrowth of YH*hspX* Δ 1 coincided with an altered host immune response, we monitored the mRNA levels of the proinflammatory cytokines TNF- α , IFN- γ , IL-6, and IL-12 in the lungs of mice infected with the WT and YH*hspX* Δ 1 strains for 2, 3, 6, and 15 weeks by semi-quantitative RT-PCR. The mRNA levels for all four cytokines were low at 2 weeks, increased to a peak after 6 weeks of infection, and declined by 15 weeks (data not shown). There were no significant differences in cytokine expression between the WT- and YH*hspX* Δ 1-infected lungs (data not shown).

Growth of *M. tuberculosis* Δ *hspX* in macrophages. In order to investigate whether the increase in the numbers of YH*hspX* Δ 1 cells in the mouse organs was due to increased growth of this strain in macrophages, we examined the survival of the mutant in murine macrophages. As shown in Fig. 3A, the number of YH*hspX* Δ 1 CFU was significantly higher than the number of WT CFU in IFN- γ -activated bone marrow-derived macrophages. Similar profiles were obtained for resting macrophages and with macrophage-like cell line J774A.1 (data not shown).

We previously showed that an *M. tuberculosis* Δ *dosR* mutant was hypervirulent in mice and activated macrophages (31). As *hspX* is under the control of *dosR*, we investigated the role of *dosR* in the increased number of CFU by reintroducing *hspX* into a Δ *dosR* mutant. It was necessary to express *hspX* from a different promoter, as the native promoter was not active in a Δ *dosR* mutant. We therefore cloned the *hspX* gene downstream of the constitutive *Ag85A* promoter in an integrating plasmid vector, introduced the plasmid (pFM171) into the Δ *dosR* mutant, and used the mutant in macrophage infection experiments. Figure 3B shows the results of infection of activated bone marrow-derived macrophages and reveals two remarkable effects. First, the increases in the numbers of CFU of the Δ *dosR* and Δ *hspX* mutants were the same in this model. Second, constitutive expression of *hspX* completely abrogated this effect. Similar results were obtained with J774A.1 macro-

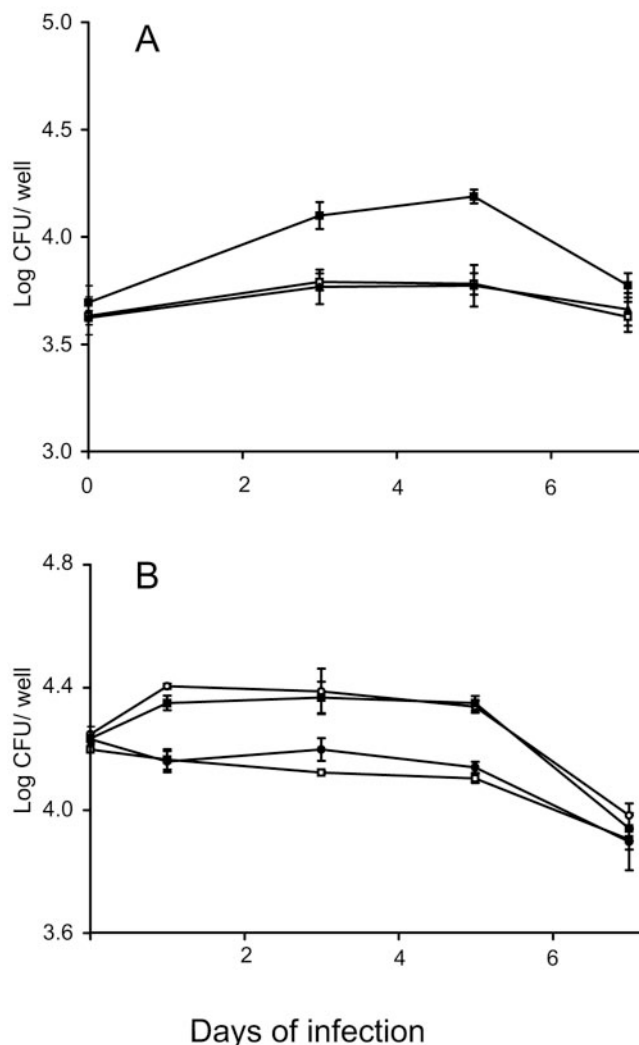


FIG. 3. Growth of *M. tuberculosis* Δ *hspX* in IFN- γ -activated macrophages. (A) Survival of the Δ *hspX* strain in bone marrow-derived macrophages. \square , H37Rv (WT); \blacksquare , Δ *hspX*; \blacktriangle , *hspX*-Comp. The values are the means and standard deviations derived from three independent experiments carried out in triplicate. The numbers of CFU of the mutant were significantly greater than the numbers of CFU of H37Rv after 3 and 5 days ($P < 0.01$ and $P < 0.001$, respectively). (B) Comparison of Δ *hspX* and Δ *dosR* mutants. \square , H37Rv (WT); \blacksquare , Δ *hspX*; \circ , Δ *dosR*; \bullet , Δ *dosR*(pFM171). The values are the means and standard deviations of two independent experiments carried out in duplicate. The numbers of CFU of the Δ *dosR* and Δ *hspX* mutants were significantly greater (as determined with a *t* test) than the numbers of CFU of the WT strain after 1, 3, and 5 days (for Δ *dosR*, $P < 0.001$, $P < 0.01$, and $P < 0.0001$, respectively; for Δ *hspX*, $P < 0.01$, $P < 0.01$, and $P < 0.0001$, respectively).

phages (data not shown). This suggests that in this model, the faster growth of the *dosR* mutant can be completely explained by the loss of *hspX* induction.

Growth of *M. tuberculosis* Δ *hspX* and complemented strains in vitro. In order to investigate if *hspX* actively slows bacterial growth, we introduced plasmid pFM171 into the *hspX* deletion mutant to obtain YH*hspX* Δ 1(pFM171) and observed the growth of this strain in an in vitro model. The WT, YH*hspX* Δ 1, YH*hspX* Δ 1(pFM171), and YH*hspX* Δ 1-comp *M. tuberculosis*

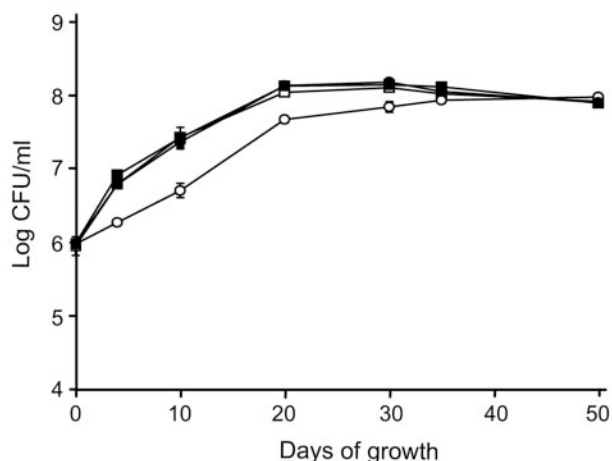


FIG. 4. Effect of constitutive *hspX* expression on axenic growth of *M. tuberculosis*. The bacilli were grown for 50 days in 7H9 medium containing 0.05% Tween 80 supplemented with 10% ADC without disturbance (51). Viability was estimated by determining the log CFU/ml at different times during incubation. □, H37Rv (WT); ■, $\Delta hspX$; ○, $\Delta hspX$ (pFM171); ●, *hspX*-Comp. The experiment was carried out twice, and similar results were obtained. The graph is a representative graph. The numbers of CFU of the WT and $\Delta hspX$ -(pFM171) strains were significantly different at 4, 10, 20, 30, and 35 days ($P < 0.0001$, $P < 0.001$, $P < 0.0001$, $P = 0.001$, and $P < 0.05$, respectively).

H37Rv strains were grown in 7H9 broth without disturbance for 50 days, and the numbers of CFU were determined at different times. As shown in Fig. 4, there was not a significant difference between the WT and the mutant. However, constitutive expression of the *hspX* gene in the mutant significantly reduced the rate of growth of the bacteria, although the growth of both strains plateaued at the same CFU level.

DISCUSSION

We clearly demonstrated that a $\Delta hspX$ mutant of *M. tuberculosis* grows more rapidly, as measured by the number of CFU, in both mouse and macrophage models. The corollary of this is that WT *M. tuberculosis* grows more slowly than the mutants, suggesting that there is either differential killing, a response to a particular host factor, or an active bacterial process that slows growth. One way to separate these possibilities is to look at axenic growth, and previous work has shown that *hspX* is associated with slow growth. Overexpression of the *M. tuberculosis hspX* gene from the powerful *hsp60* promoter resulted in reductions in the growth rates of both *Mycobacterium smegmatis* and *M. tuberculosis* (53). In *M. smegmatis* this was attributed to an increased lag phase, while in *M. tuberculosis* the logarithmic growth was slower and the amount of autolysis was greatly reduced. In our experiments, constitutive expression of *hspX* also led to a decreased growth rate for *M. tuberculosis*. These studies, combined with the other work described here, strongly suggest that WT *M. tuberculosis* actively slows its growth in macrophages and in mice and that HspX plays a central role.

It is tempting to link this slow growth to the latency that is such a key aspect of the pathogen's lifestyle and to the active

participation of the bacterium in granuloma formation (7). However, if these characteristics are related, then the process starts as soon as the bacteria enter cells. The in vivo studies showed that this is an early effect, visible after 7 days of infection, long before growth of both WT and mutant bacteria ceased after 3 weeks. This contrasts with indications that *hspX* expression and the plateauing of the number of CFU seen with the onset of the adaptive immune system are coincident (44). An alternative explanation is that we observed a mechanism (unrelated to latency) by which *M. tuberculosis* avoids detection by the immune response in order to increase the chance of establishing an infection, possibly not involving the high levels of HspX that accumulate later in infection. The difference in the growth rates in vivo between the WT and $\Delta hspX$ strains was not mirrored in axenic growth, although exponential growth was observed in both conditions. This indicates that the growth conditions were different and that we examined only one factor in a complex interaction.

A different *hspX* mutant ($\Delta hspX::hpt$), which showed reduced growth in macrophages, was described previously (54). The apparent contradiction between the previous report and our results may be explained by the fact that in the $\Delta hspX::hpt$ mutant (constructed before the genome sequence was available) a 1,026-bp EcoRV deletion removed not only the *hspX* gene but also the first 405 bp of the downstream gene Rv2030c and 176 bp of the upstream DNA, which contains the divergent *acg* promoter (Fig. 1A). In addition to directly affecting these three genes, there may be polar effects on the other two genes in the *hspX* operon, *pfkB* and Rv2028c. As no complementation with the *hspX* gene was carried out in the previous study, we suggest that one or more of the other genes inactivated in this construct were responsible for the attenuation phenotype.

We demonstrated that higher numbers of mutant bacilli (approximately 10-fold higher) than of WT cells are present in tissues, as determined both by counting CFU and by direct microscopy. It is usually assumed that the numbers of CFU and pathology are linked, with increases in one leading to increases in the other. We saw some increased pathology, and the spleens were enlarged, but we expected to see a greater difference than we did and the profiles of the four cytokines that we tested were similar. Thus, an *mce1* mutant (45) grew more rapidly (approximately 1 log) than the WT strain in mouse lungs, but there were more pronounced changes in both pathology and TNF- α production than we observed. Other work has shown that CFU and pathology can be disengaged. North et al. (26) showed that the same number of CFU of three different strains resulted in different rates of mortality, while the numbers of cells of clinical isolate HN878 of *M. tuberculosis* in the tissue of immune-competent mice were higher than the numbers of cells of less virulent isolates, yet this isolate does not cause more severe tissue damage, possibly due to a reduced Th1 response (23). Conversely, "immunopathology" mutants have been isolated where WT levels of CFU were observed, but they have greatly reduced pathology (18, 48). We suggest that these reports provide an opportunity to separate different aspects of the host and pathogen biology in this complex disease, where host pathology may be advantageous in some stages of the infection but not in other stages.

The observation of hypervirulence in *M. tuberculosis* mutants, whether measured by the number of CFU or by in-

creased pathology, is relatively new, and hypervirulence has been observed only rarely in other pathogens (8). Mutations in five structural genes (24) and four 2CSs (*dosR*, *kdpDE*, *trcS*, and *trcXY*) (31) caused hypervirulence in a SCID mouse model. In immunocompetent mouse models, a strain lacking *mce1* was also shown to be hypervirulent, as measured by earlier mortality, while *dosR* and *pknH* mutants showed increased growth (30, 31, 45). The phenomenon in all these organisms involves more rapid growth at very early stages of infection before the acquired immune response is established. These findings suggest that slowing down the growth of WT *M. tuberculosis* in vivo is an integral part of the infection process that involves a large number of genes.

Our previous finding that the *M. tuberculosis* Δ *dosR* mutant grows more rapidly in SCID and DBA mice and in macrophages (31) is contradicted by a report which showed that a different *dosR* mutant was attenuated in guinea pigs and in resting human monocytes (22). The differences could be explained by the models (for example, the guinea pig is much more susceptible than the mouse to tuberculosis), the mutation (we used an unmarked deletion, and Malhotra et al. used a Kan^r insertion), or the strain (although we and Malhotra et al. used H37Rv, it is likely that substrains have developed around the world). There is also a conflict with the essentiality data generated using the TrasH methodology (41). In this methodology, populations of transposon mutants are used to infect mice and recovered from the lungs, and relative growth is measured using microarrays. Although the methodology was designed to see essentiality and attenuation (by reduced representation of the relevant mutant), hypervirulence should be visible through increased representation. Surprisingly, no real hypervirulence has been seen in any of the genes in cases in which mutants have been reported by us or other workers to be hypervirulent. One possible explanation for this discrepancy is that the hypervirulence is a recessive effect and infection by a mixture of strains abrogates this effect. Our hypothesis that HspX actively causes a slowing of growth is consistent with this explanation, as HspX has been reported to be present in culture supernatants (16) (see below).

There have been studies in which the workers have looked at different levels of virulence in clinical isolates (28, 38), and it is possible that differences in hypervirulence genes may play a part in this variation. Small deletions have been identified in clinical isolates using microarrays (11, 17), but the isolates that have been characterized do not have detectable deletions in any of the hypervirulence genes identified so far (although some *dosR* regulon genes are deleted). In one recent study the workers looked at hypervirulence in Beijing strains and showed that this hypervirulence was linked to the presence of a particular glycolipid (37). This is a completely different phenomenon than the one described here, in which loss of a gene increased virulence.

Of the 2CS mutants that we described previously (31), the Δ *dosR* mutant was the most hypervirulent mutant. The data presented here show that although DosR activates approximately 50 genes, in macrophages at least the increase in the number of CFU seen with the Δ *dosR* strain can be completely explained by the lack of HspX expression. However, this explanation is not definitive, as the situation could be complicated by an active effect of *hspX* on the growth rate that masked

the involvement of other genes. We mention above that *dosR* is activated in vitro by hypoxia; it has also been shown to respond to other signals, including NO and ethanol (19, 27, 49). Indeed, macrophage experiments have shown that the *dosR* regulon is activation specific and NO dependent (42), indicating that inside the cells, NO is the signal. We found no obvious reduction in survival in the presence of NO for the *hspX* mutant compared to the WT strain, suggesting that the *dosR* gene does not appear to play a role in protection against NO in the conditions that we used. Other members of the regulon have been identified as probable nitroreductases (*acg*, Rv3127, and Rv3131) (36), and these genes are more likely candidates. We suggest that DosR-induced genes play different roles; some protect the bacteria from the host response, while others initiate slow bacterial growth.

The HspX protein has been shown act as a chaperone (3, 53). There was no difference in survival of the *hspX* mutant at 55°C, suggesting that *hspX* is not essential for protection of the bacterium against these heat shock conditions. As *hspX* is only one of the heat shock-like genes in *M. tuberculosis*, the other genes may play a more important role. Not only is the α -HSP family generally just one part of a multichaperone network (25), but there is also a second α -HSP in *M. tuberculosis* (*acr2*) (47), so there may be functional redundancy. Indeed, it is difficult to envisage how the hypervirulence phenotype that has been observed could be related to HspX chaperone activity, suggesting that, as is seen in other α -HSPs (25), HspX has more than one function.

The identification by Lee et al. (20) of HspX as the major membrane protein of *M. tuberculosis* suggests that HspX does not function purely cytoplasmically. HspX has been shown to accumulate in the cell wall in long-term stationary-phase cultures of *Mycobacterium bovis* BCG (5); concomitant with this was a marked thickening of the cell wall, although it was not shown how these facts were related. More recently, proteome analysis has identified HspX in both whole-cell lysates and culture filtrates of *M. tuberculosis* (16, 39). HspX has no classical signal sequence, but other secretion pathways have been shown to operate in *M. tuberculosis* (1). The CotM protein of *Bacillus subtilis* is an α -HSP that acts as an integral part of the spore coat, possibly due to cross-linking by a transglutaminase (10). While HspX could still function as a chaperonin outside the cytoplasm, it is tempting to speculate that it also plays a structural role in the *M. tuberculosis* cell wall and that this directly or indirectly affects the growth rate of the bacterium. If the slowing down of growth is related to latency, then it is remarkable that this process (usually associated with the late stationary phase) starts so soon after infection. What is clear is that the results of this work and other studies strongly support the notion that HspX is critically involved at different stages of infection.

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