Charaterization of a Novel Heat Shock Protein (Hsp22.5) Involved in the Pathogenesis of Mycobacterium tuberculosis

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Tuberculosis is a worldwide health problem, given that one-third of the world’s population is currently infected with Mycobacterium tuberculosis. Understanding the regulation of virulence on the molecular level will provide a better understanding of how M. tuberculosis can establish chronic infection. Using in vivo microarray analysis (IVMA), we previously identified a group of genes that are activated in BALB/c mouse lungs compared to in vitro cultures, including the rv0990c gene. Our analysis indicated that this gene is a member of the heat shock regulon and was activated under other stress conditions, including survival in macrophages or during the late phase of chronic tuberculosis in the murine lungs. Deletion of rv0990c from the genome of M. tuberculosis strain H37Rv affected the transcripational profiles of many genes (n = 382) and operons involved in mycobacterial survival, including the dormancy regulon, ATP synthesis, respiration, protein synthesis, and lipid metabolism. Comparison of the proteomes of the mutant to those of the wild-type strain further confirmed the differential expression of 15 proteins, especially those involved in the heat shock response (e.g., DnaK and GrpE). Finally, the rv0990c mutant strain showed survival equivalent to that of the isogenic wild-type strain during active tuberculosis in guinea pigs, despite showing significant attenuation in BALB/c mice during the chronic phase of the disease. Overall, we suggest that rv0990c encodes a heat shock protein that plays an important role in mycobacterial virulence. Hence, we renamed rv0990c heat shock protein 22.5 (hsp22.5), reflecting its molecular mass.

Tuberculosis is a devastating disease that threatens one-third of the world’s population, with 1.8 million deaths each year from infection with Mycobacterium tuberculosis (11). Following aerosol infection, M. tuberculosis can persist inside the host macrophages for long periods, leading to chronic infection. Under certain circumstances, chronically infected patients can develop an active progressive infection that could lead to death (6). Unfortunately, several aspects of the chronic phase of tuberculosis are not completely understood. For example, the molecular basis responsible for the persistence of M. tuberculosis inside the host remains largely unknown. Also, the triggers for M. tuberculosis reactivation are not well characterized. Several in vitro and in vivo models have been developed in order to elucidate the mechanisms employed by the pathogen to survive and persist inside the host (5, 12, 25, 30–33). Such studies have identified genes activated during M. tuberculosis survival inside macrophages or under hostile stress conditions, including transcriptional regulators which control the expression of a large number of genes (1, 22). One such gene is rv0990c, which was preferentially activated in murine lungs among a group of 32 genes sharing the same genomic locus, termed the in vivo-expressed genomic island, iVEGI (31). The iVEGI includes several genes and operons that are involved in mycobacterial pathogenesis and persistence in the host, such as the cso operon, rv0971c, and mprAB (34, 35). In this report, we provide more insights into the roles played by rv0990c in tuberculosis pathogenesis on both cellular and host levels.

Earlier analysis indicated that expression of rv0990c was upregulated upon exposure to heat shock among other members of the heat shock regulon (28). Members of the heat shock responsive genes typically play an important role as chaperons in protein folding, assembly, transport, and degradation, especially under stress conditions (21). In another report, progressive hypoxia identified transcripts of rv0990c among 230 other hypoxia responsive genes (24). Although the exact function of the gene remains to be elucidated, the presence of such a gene among the 230 hypoxia genes can suggest a role for hypoxia-responsive genes in mycobacterial persistence. In this study, we identified rv0990c as a new member of the heat shock regulon and showed the unique transcriptional and translational profiling of the rv0990c mutant in comparison to the wild type, the H37Rv strain. We also identified triggers that activate rv0990c gene transcripts. Finally, we demonstrated that rv0990c (here designated heat shock protein 22.5 [hsp22.5]) has little or no impact on the progression of active tuberculosis in guinea pigs. However, it has a significant impact on the survival of M. tuberculosis during the chronic phase of tuberculosis in mice.

MATERIALS AND METHODS

Strains, media, and plasmids. Escherichia coli DH5α and HB101 were used as host cells for cloning purposes in all experiments presented here. M. tuberculosis
H37Rv and Mycobacterium smegmatis mc2155 strains were grown in Middlebrook 7H9 liquid medium and on Middlebrook 7H10 plates supplemented with albumin dextrose catalase (ADC) and antibiotics, when needed (25 μg/ml kanamycin or 50 μg/ml hygromycin). Protocols for DNA manipulations employed throughout this report, including PCR, cloning, DNA ligations, and electroporation were as described previously (8). Total RNA samples were extracted from mycobacterial cultures grown to an optical density at 600 nm (OD600) of 0.5 or 1.5 using a Trizol-based protocol (Invitrogen, Carlsbad, CA) as described previously by our group (29, 30). Isolated mycobacterial total RNA samples were treated with DNase I (Ambion, Austin, TX) until no DNA was detected using PCR primers for the 16S rRNA gene. A list of primers used in this report is presented in the supplemental material.

Generation of new mycobacterial constructs. A specialized transduction protocol was adopted with a few modifications to delete the rv0990c gene using the virulent strain of M. tuberculosis (4). Briefly, approximately 800-bp fragments flanking the rv0990c sequence were amplified. Amplicons were cloned into pGEM-T vector (Promega, Madison, WI), and the sequence was verified before ligation into the pYUB845 vector using SpeI and HindIII for the left arm and Xbal and Acc65I for the right arm. The generated cosmids was packaged into viable mycobacteriophages in M. smegmatis using an in vitro packaging system (GigaPack III Gold from Stratagene). Temperature-sensitive full phages with the rv0990c and rv0992c deletion mutated were used to transduce M. tuberculosis cells prepared as described previously. Following 4 to 6 weeks of incubation at 37°C, hygromycin-resistant colonies were selected for further analysis. PCR and Southern blot analyses were used to verify the mutant genotypes as described previously by our laboratory (1). For complementation experiments, the coding sequence of rv0990c was amplified, cloned into pGEM-T vector, and verified by DNA sequencing. The amplified products were doubly digested with HindIII restriction enzymes, and their inserts were gel purified before ligation into the integrative mycobacterial shuttle vector pMv361 (15) to give rise to pML30 vector in which rv0990c is under the control of the hsp65 promoter. The resulting vector pML30 was electroporated into electrocompetent M. tuberculosis cells. Transformants were selected and subsequently analyzed by PCR to verify integration of the delivered sequences into the M. tuberculosis genome.

For luc2 assays in recombinant M. smegmatis, the intergenic regions of the rv0990c-rv0992c loci were cloned, sequenced, and ligated into pCY77 vector. The luc2 assays were performed in the soluble fractions of recombinant M. smegmatis in triplicates and repeated at least twice (1).

Animal infections. For the guinea pig infections with M. tuberculosis strains, female Hartley guinea pigs (250 to 300 g; Charles River) were housed in a biosafety level 3 (BL-3) environment and received water and chow ad libitum. The animals were maintained according to protocols approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine. Separate groups of 12 guinea pigs were aerosol infected using a Madison chamber aerosol generation device (College of Engineering Shops, University of Wisconsin, Madison, WI) calibrated to deliver approximately 100 bacilli into guinea pig lungs (10). Cultures of M. tuberculosis H37Ra and H37Rv strains were grown to mid-log phase (OD600 ≤ 0.5) and were doubly digested with HindIII and HincII restriction enzymes, and their inserts were gel purified before ligation into the integrative mycobacterial shuttle vector pMv361 (15) to give rise to pML30 vector in which rv0990c is under the control of the hsp65 promoter. The resulting vector pML30 was electroporated into electrocompetent M. tuberculosis cells. Transformants were selected and subsequently analyzed by PCR to verify integration of the delivered sequences into the M. tuberculosis genome.

For qRT-PCR, cDNA was synthesized from 1 μg of total RNA using SuperScript III (Invitrogen) as directed by the manufacturer, in the presence of SYBR green and 250 ng of mycobacterial genome-directed primers. SYBR green qRT-PCRs were performed using gene-specific primers (see the supplemental material) at a concentration of 200 nm. The cycle conditions were as follows: 95°C, 30 cycles, and 40 cycles of 95°C for 15 s and 60°C for 30 s. Quantitative RT-PCR samples were analyzed in triplicates, the threshold cycle values were normalized to levels of 16S rRNA transcripts, and fold changes were calculated by the threshold cycle (ΔΔCt) method.

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For Western blot analysis, acidified mycobacterial proteins were solubilized, denatured in 20 μl of 50 mM HEPES, pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 20% glycerol, and 10% β-mercaptoethanol for 30 min and then subjected to 10% SDS-PAGE and stained with Coomassie blue. Western blots were probed with antibodies specific to chaperonin and to lipoarabinomannan (LAM). Stained bands were quantified using ImageJ software (NIH). For in vivo quantitative real-time PCR (qRT-PCR), we used RNA isolated from an additional mouse group utilized previously for DNA microarray analysis by our group (32).

Growth in macrophages. Following expansion, THP-1 cells were centrifuged at 400 × g for 10 min and allowed to differentiate by the addition of 20 nM phorbol 12-myristate 13-acetate (PMA). The infection of the monolayer-adhering cells with the M. tuberculosis H37Rv strain was performed to achieve an MOI of 1:10 (cell:bacteria). After 3 h of infection, cells were washed with sterile PBS buffer and incubated at 37°C in 5% CO2 with RPMI medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 24 h. Cells were lysed with 0.05% sodium dodecyl sulfate (SDS), and bacteria were collected and processed for RNA extraction using the Trizol method (1).

Stress treatments of M. tuberculosis. M. tuberculosis cultures were grown to mid-log phase (OD600 = 0.5), and their colony counts were determined by plating on Middlebrook 7H10 agar. Subcultures (10 ml each) were subjected to 0.05% SDS treatment or H2O2 (10 mM) for 4 h at 37°C or heat shock at 45°C for 30 min or 1 h at 37°C untreated in a slow-shaking incubator. These cultures were plated and harvested for RNA extraction as described above.

DNA microarrays. Mycobacterial cultures growing at early stationary phase (OD600 = 1.5) were used for RNA isolation (29). Before DNA microarrays, double-stranded cDNA (ds-cDNA) was synthesized from 10 μg of total RNA using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) as directed by the manufacturer, in the presence of 250 ng genome-directed primers. The ds-cDNA was cleaned up and labeled following the NimbleGen gene expression analysis protocol (Molecular Devices Corporation, Sunnyvale, CA). The NimbleGen hybridization buffer and commercial hybridization chambers (TeleChem International, Inc., Sunnyvale, CA) overnight at 42°C. Following hybridization, washing steps were performed using NimbleGen washes I, II, and III as recommended by the manufacturer. Slides were scanned using an Axon GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA), and fluorescent intensity levels were extracted using NimbleScan (Nimblegen) and normalized to a mean value of 1,000. Determination of significantly changed genes was performed using a flexible empirical Bayes model, specifically, the LNN model in the EBarrays package employing an R language (http://www.bionconductor.org). A cutoff of 0.9 for the probability of differential expression (PDE > 0.9) was used to determine significantly changed genes.
tberculosis strain H37Rv amino acid sequence database using the in-house Sequest search engine, with methionine oxidation as a variable modification. Data were subsequently processed by Scaffold, version 2.06 (Proteome Software, Inc.) to yield unambiguous protein identifications with at least two unique peptides per protein. Only protein identifications with a probability of at least 95% were considered for further analysis.

Statistics. Unless indicated otherwise, data generated in our study were expressed as the mean ± standard error of the mean (SEM). Student's t test was used to assess the significances of differences in the number of CFU between groups.

RESULTS AND DISCUSSION

Genetic organization of the rv0990c region. The rv0990c gene is located in the previously identified in vivo-expressed genomic island (IVEGI) (31). Protein sequence analysis of the rv0990c gene indicated that it encodes a 218-amino acid polypeptide with a molecular mass of 22.5 kDa. Further sequence analysis indicated that the rv0990c gene is highly conserved among all sequenced members of the genus Mycobacterium, with 63 to 100% sequence identity on the amino acid level (see the supplemental material). Compared to members outside the Mycobacterium genus, the sequence identity drops to less than 50%. In addition, similar to a large family of heat shock-responsive proteins, a CIRCE-like element (20) was found 28 bp upstream of the predicted initiation codon of rv0991c, given the putative translational start site of this downstream gene. It was suggested that rv0990c and the 2 flanking genes were organized as a single operon (23). Using RNA isolated from heat shock cultures (45°C for 1 h), the reverse transciptase PCR (RT-PCR) experiments showed the transcription of the rv0989c-rv0990c and rv0990c-rv0991c junction regions but not the rv0991c-rv0992c fragments (Fig. 1A and B), confirming the operon structure of the 3 genes. The same operon organization was not clear when RNA samples from untreated cultures were used (data not shown). In fact, sequence analysis of the DNA region encompassing the rv0990c gene (rv0989c-rv0992c) showed the presence of intergenic regions of about 60 to 75 bp between each gene, suggesting independent gene transcriptions.

To test the possibility of the presence of a promoter in these intergenic regions, the 4 intergenic regions were amplified by PCR and cloned into a promoterless lacZ vector (pCV77) and electrotoprinated into M. smegmatis mc2155 for evaluating the expression of LacZ. All transformants developed blue colors on X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) plates except the negative controls (empty vector and pCV77 harboring the dif fragment of rv0990c [see the supplemental material]). Blue clones representing the 4 intergenic regions were assayed for the expression of LacZ in liquid cultures of M. smegmatis mc2155 for evaluating the expression of lacZ. All transformants developed blue colors on X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) plates except the negative controls (empty vector and pCV77 harboring an internal fragment of rv0990c [see the supplemental material]). Blue clones representing the 4 intergenic regions were assayed for the expression of lacZ. DNA was used as a positive control, while RNA was used as the negative control for each intergenic region. Additionally, primers internal to hsp22.5 were used as a positive control for cDNA amplification. (C) ß-Galactosidase activity in recombinant M. smegmatis harboring the different constructs encoding lacZ under the control of variable putative promoter regions. ß-Galactosidase activity was performed in soluble fractions of M. smegmatis lysates, and experiments were repeated at least twice in triplicates.

Expression of rv0990c under different stress conditions. Because rv0990c is induced in mouse lungs (31), we investigated the expression of this gene under different stress conditions, including SDS, H2O2, and high temperature (45°C) as well as in stationary-phase cultures. Quantitative RT-PCR (qRT-PCR) analysis showed that rv0990c was modestly upregulated upon exposure to H2O2 and SDS (2.5- and 2.8-fold change, respectively) compared to untreated M. tuberculosis cultures and almost unchanged in the stationary phase of bacterial growth (Fig. 2A). Interestingly, both rv0990c and rv0991c transcripts were highly upregulated upon exposure to high temperature (45°C) for 30 min and 1 h (33- and 80-fold change, respectively) compared to same culture grown at 37°C (Fig. 2B). It is noteworthy that although the expression of rv0990c was transient, the expression of dnaK and grpE (key members of the heat shock regulon) were upregulated after short exposure to high temperature and remained high even after 24 h (see the supplemental material), suggesting a specific role for rv0990c during heat shock response. Accordingly, we renamed rv0990c as heat shock protein 22.5 (hsp22.5) to reflect its estimated molecular mass.

In our earlier report (32), transcripts for hsp22.5 were higher in murine lungs at 145 days postinfection than at 20 days
FIG. 2. Quantitative real-time PCR analysis of hsp22.5 under variable stress conditions. (A) The profile of hsp22.5 transcripts was analyzed following culture (OD$_{600}$ = 0.5) exposure to 10 mM H$_2$O$_2$ or 0.05% SDS independently and when mid-log phase cultures were compared to stationary-phase cultures (OD$_{600}$ = 1.5). (B) The transcriptional profile of hsp22.5 and other heat shock genes (rv0991c, dnaK, and grpE) following heat shock exposure for 30 min (black bar) or 1 h (gray bar). (C) The profile of hsp22.5 transcripts following THP-1 infection for 24 h or survival within murine lungs. Fold change of every sample was compared to that of untreated M. tuberculosis cultures growing to OD$_{600}$ of 0.5. Results are representative of two biological replicates.

postinfection. To better analyze conditions that trigger the activation of hsp22.5, we examined the expression of hsp22.5 on both cellular and organ levels. Transcripts for hsp22.5 were slightly more abundant upon infection of THP-1 macrophages (2.5-fold changes) than in in vitro culture (Fig. 2C). In earlier reports, the hsp22.5 transcripts did not change significantly inside human peripheral blood mononuclear cells (PBMCs) or in THP-1 macrophages after 7 days and 24 h of infection, respectively (9, 14). Interestingly, in our hands the modest activation observed reached much higher levels (77- and 92-fold) in murine lungs at 145 and 220 days postinfection (compared to in vitro cultures), respectively, suggesting a role for hsp22.5 during the chronic stage of tuberculosis (Fig. 2C). Overall, transcriptional analysis showed that hsp22.5 is selectively upregulated during murine infection and heat shock but to a lesser extent in other stress conditions (e.g., oxidative and alkaline stress). However, such a profile suggests the involvement of hsp22.5 in mycobacterial persistence.

Deletion of hsp22.5 from the genome of M. tuberculosis. The hsp22.5 coding sequence was replaced by a hygromycin cassette using a protocol for homologous recombination (4). The genotype of the obtained mutants (Δhsp22.5) was verified by PCR and Southern blot technique (Fig. 3). Briefly, two specific probes were used in Southern blot verification of the genotype of the mutant. One probe was hygromycin specific, and the other was from the missing coding sequence of hsp22.5. Both probes gave the expected hybridization patterns. The RT-PCR analysis of the H37Rv and Δhsp22.5 mutant cultures (OD$_{600}$ of 1.5) revealed the transcription of the up- and downstream genes of hsp22.5, excluding the possibility of a polar effect of the hsp22.5 deletion. One of the mutants was used for subsequent complementation analysis using integrative vector pMV361. The expression of hsp22.5 was verified in the complementation strain (the Δhsp22.5::hsp22.5 strain) by qRT-PCR (data not shown). Additionally, we examined the survival of the Δhsp22.5 mutant in Middlebrook 7H9 broth, after exposure to heat shock or following infection of naive and activated J774A.1 cells. In all of these cases, there were no significant differences between the mutant and the wild-type isogenic parent strain, suggesting a limited role for hsp22.5 in survival under in vitro conditions and inside macrophages. The obtained CFU counts following J774A.1 infection are provided in the supplemental material.

Δhsp22.5 mutant is attenuated during chronic tuberculosis. In order to examine the contribution of hsp22.5 to M. tuberculosis pathogenesis, we infected groups of guinea pigs with comparable doses of H37Rv or the Δhsp22.5 or Δhsp22.5::hsp22.5 strains. During the first 21 days after infection, the H37Rv and Δhsp22.5::hsp22.5 strains showed typical exponential growth, reaching a peak lung burden of 10$^6$ CFU/lung for each strain. On the other hand, the Δhsp22.5 mutant showed a mild initial growth defect relative to the wild type (<1 log difference) (see the supplemental material). At day 42 after infection, following the onset of adaptive immunity in guinea pigs, the lungs of animals infected with the Δhsp22.5 mutant harbored fewer bacilli than those infected with H37Rv (4.80E + 05 and 1.08E + 06 CFU/lungs) for each group, respectively. However, this difference was not statistically different (P = 0.08). Moreover, gross examination of lungs infected with all strains at day 42 after infection revealed discrete tubercle lesions distributed...
throughout the lung surface for guinea pigs infected with the 3 strains (data not shown). Histological examination of day 42 lung samples showed no significant difference in inflammation between groups. Since the chronic phase of tuberculosis in guinea pigs is not completely characterized, we opted to test the impact of hsp22.5 on this phase using the standard murine model of tuberculosis.

Aerosol-challenged BALB/c mice with comparable doses of the H37Rv, Δhsp22.5, or Δhsp22.5::hsp22.5 strains showed a pattern of growth during the active phase of tuberculosis, similar to the one obtained with guinea pigs. However, after 8 weeks postinfection, the bacterial load of the mutant strain (the Δhsp22.5 mutant) continued to decline in number until the end of the experiment at 38 weeks, with a significant reduction in the colonization level (P < 0.02), unlike groups infected with H37Rv and complemented strains (Fig. 4A). More analysis of the CFU count at the time of death confirmed the higher level of colonization in the H37Rv-infected group than in the Δhsp22.5 mutant-infected group (see the supplemental material). Interestingly, the survival curve of infected mice confirmed the Δhsp22.5 mutant attenuation, with a median survival time (MST) for Δhsp22.5 mutant-infected mice of 52 weeks compared to an MST of 28 and 40 weeks for the H37Rv- and Δhsp22.5-complemented strain-infected animals, respectively (Fig. 4B). This intermediate survival curve for the complemented strain could be attributed to the expression level of hsp22.5 under the hsp65 constitutive promoter rather than its native promoter. Histopathology of mouse lungs (Fig. 4C) displayed lower levels of granuloma formation (score 2), but similar levels of lymphocyte infiltration in the Δhsp22.5 mutant-infected mice compared to those infected with H37Rv and complemented strains (score 5) (see the supplemental material). Overall, both colonization and histopathology data suggested a role for hsp22.5 in M. tuberculosis virulence, especially at the chronic phase of tuberculosis. Since our model of guinea pig infection did not reach the chronic phase, the attenuation phenotype was observed only with mice.

Global transcriptional profile of Δhsp22.5. Earlier predictions of the function of hsp22.5 included the encoding of a transcriptional regulator based on bioinformatic sequence analysis (32). However, electromobility shift assays failed to show the binding ability of Hsp22.5 to any of the 10 examined putative promoter fragments (data not shown), suggesting an indirect mechanism for regulating other genes if hsp22.5 would play any regulatory role. To examine this possibility, we used DNA microarrays to compare the mycobacterial transcriptional profiles in the presence and absence of hsp22.5 (1). Replicate microarray hybridizations (n = 3) were performed from 2 biological samples with a high correlation level (R = 0.8) among replicates. Using Bayesian statistics, we identified significantly regulated genes with a probability of differential expression (PDE) value of >0.9 and a >±2-fold change. Analysis of the transcriptional profiles of the mutant versus the H37Rv strain revealed a significant number of regulated genes.

FIG. 3. Deletion of the hsp22.5 coding sequence from the genome of M. tuberculosis H37Rv strain. (A) A sketch drawn to scale displaying the sequence coding for hsp22.5 before and after replacement by the hygromycin cassette (hygR) as detailed in Materials and Methods. BamHI restriction sites flanking hsp22.5 are shown, and a graphical omission of 4.7 kb, as indicated by hash marks, was made. (B) Southern blot confirmation of the deletion. Genomic DNA samples from both the wild type (H37Rv) and isogenic mutant (the Δhsp22.5 mutant) were digested with BamHI and probed using an hygR-specific probe for panel 1 or an hsp22.5-specific probe for panel 2. Two mutants were examined for the results depicted for each panel. (C) Reverse transcriptase analysis of transcripts of hsp22.5 and neighboring genes in cultures of the H37Rv and Δhsp22.5 strains.
Among the identified transcriptome, only 67 genes were slightly induced in the Δrv0990c mutant, while the majority (n = 315) were repressed. To verify the obtained transcriptomes, we performed qRT-PCR using the cDNA from the same RNA samples isolated from H37Rv, the Δhsp22.5 mutant, and the Δhsp22.5::hsp22.5 strain and used for DNA microarrays (see the supplemental material). Overall, there was an agreement between the changes in transcript levels between qRT-PCR and DNA microarrays for all repressed genes (n = 9) in the Δhsp22.5 mutant (see the supplemental material).

However, transcripts of the induced genes (n = 3) in the Δhsp22.5 mutant were not verified by qRT-PCR. With an overall 75% agreement between transcripts measured by DNA microarrays and qRT-PCR, we focused our subsequent analysis on the group of genes that were regulated in the absence of Hsp22.5.

The global changes of repressed genes included several key pathways for M. tuberculosis survival during infection (virulence genes, dormancy regulon, ATP synthesis, respiration, protein synthesis, and lipid metabolism). Several of the affected genes encoded essential proteins, such as DnaK and...
GrpE. Both genes encoding these proteins are among operons that are under the negative control of HspR (28). Among the affected genes in the Δhsp22.5 mutant, 10 genes were among the heat shock regulon (28), 16 were transcriptional factors, and 18 were hypoxia genes (see the supplemental material). Additionally, the genes encoding DevR and DevS were also downregulated in the mutant and consequently so were most of the dosR-dependent genes, such as 

\[ \text{atpD} \] 

and 

\[ \text{atpE} \]

(26) or ATP synthesis (e.g., 

\[ \text{atpF} \]

and 

\[ \text{atpH} \]

and 

\[ \text{atpD} \]

were among the downregulated genes. Genes involved in cell invasion, such as 

\[ \text{mce1 operon} \]

and the 

\[ \text{esat6 and cfp10} \]

(18) genes were also downregulated, suggesting a role for 

\[ \text{rv0990c} \]

in 

\[ \text{M. tuberculosis} \]

virulence. The whole-transcriptome levels are listed in the tables in the supplemental material, and only a partial list of essential genes is shown in Table 1. Overall, the profiled transcriptome for the 

\[ \text{Δhsp22.5} \]

mutant suggested the involvement of 

\[ \text{hsp22.5} \]

in the transcription of a large number of genes, including those involved in 

\[ \text{M. tuberculosis} \]

survival and persistence. It is possible that the observed mutant phenotype is caused by the regulation of any combination of genes influenced by the presence of 

\[ \text{hsp22.5} \].

### Proteomics analysis

The significant changes observed in the transcriptomes of the 

\[ \text{Δhsp22.5} \]

mutant constituted a strong rationale to examine such changes on the proteomic level. Protein pellets from cell extracts of both the H37Rv and 

\[ \text{Δhsp22.5} \]

strains were subjected to tryptic digestion followed by nanoLC-MS/MS. Recently a similar work used such a technique in which approximately 2,000 proteins were identified and only 3 proteins were found to be differentially expressed between H37Rv and the 

\[ \text{ΔclgR} \]

(a mycobacterial regulator) mutant (13). Approximately 1,853 proteins (46% of total proteome) were identified in two biological samples of each strain (see the supplemental material). Among this list of genes, we were able to identify the downregulation of 15 proteins in agreement with the microarray data, including the 

\[ \text{dnaK} \]

and 

\[ \text{grpE} \]

proteins (Table 2). Other identified proteins include the 

\[ \text{85C antigen} \]

, a member of 85 complex antigens that is involved in cell wall biosynthesis, mycobacterial pathogenesis, and immune system modulation (7). On the other hand, proteomic comparison revealed some proteins with differential expressions that are inconsistent with the transcriptional profiles identified previously (e.g., HspX, ESAT6, and Mas [mycocer- osic acid synthase]) (3). Such observations could be attributed to the regulation of those genes on the translational rather than the transcriptional level in addition to the sensitivity of the used proteomic approach. Previously, disparity between mRNA and protein levels for the 

\[ \text{hspX} \]

gene, a prominent member of the dosR regulon, was noticeable (17), suggesting regulation on the translational level. A similar scenario could be proposed here. Moreover, some of the proteins undetectable by LC could be attributed to their low levels of expression which were masked by the presence of other proteins (e.g., the 

\[ \text{Ag85 complex} \]

) with much higher levels of expression. Additional analysis of the transcriptome and proteome of intracellular bacilli of the 

\[ \text{Δhsp22.5} \]

mutant is warranted to further characterize genes participating in general stress responses during chronic tuberculosis.

### Conclusion

The role of heat shock response genes in mycobacterial survival is not completely elucidated, despite evidence of its wide impact on the survival of other bacterial pathogens (21). In this work we examined the involvement of a novel heat shock protein that we named 

\[ \text{hsp22.5} \]

in mycobacterial persistence in BALB/c mice, despite the precise function and role of 

\[ \text{hsp22.5} \]

remaining largely unknown. Testing of the virulence of the 

\[ \text{hsp22.5} \]

mutant in guinea pigs using a small aerosol dose will further improve our understanding of the role of this gene in chronic tuberculosis. Both microarrays and

### Table 1. List of mycobacterial operons downregulated in cultures of the Δhsp22.5 mutant compared to the H37Rv strains by use of DNA microarrays

<table>
<thead>
<tr>
<th>Gene ID or cluster</th>
<th>Possible function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0166-Rv0178</td>
<td>mce1 operon involved in cell invasion</td>
</tr>
<tr>
<td>Rv0666-Rv0668</td>
<td>Involved in DNA transcription</td>
</tr>
<tr>
<td>Rv0824c-Rv0824c</td>
<td>Lipid metabolism</td>
</tr>
<tr>
<td>Rv1195c-Rv1198</td>
<td>PE family and Esat6-like proteins</td>
</tr>
<tr>
<td>Rv1297</td>
<td>rho (transcriptional terminator)</td>
</tr>
<tr>
<td>Rv1594c-Rv1596</td>
<td>Possible involvement in quinolinate biosynthesis</td>
</tr>
<tr>
<td>Rv1641-Rv1643</td>
<td>Protein synthesis</td>
</tr>
<tr>
<td>Rv1736c-Rv1738</td>
<td>Members of dormancy regulon</td>
</tr>
<tr>
<td>Rv2007c-Rv2017</td>
<td>Members of dormancy regulon</td>
</tr>
<tr>
<td>Rv2020c-Rv2032</td>
<td>Members of dormancy regulon</td>
</tr>
<tr>
<td>Rv2245c-Rv2247</td>
<td>AcpM, KasA, KasB, and AccD6 (fatty acids biosynthesis)</td>
</tr>
<tr>
<td>Rv2346c-Rv2348c</td>
<td>Esat6-like proteins</td>
</tr>
<tr>
<td>Rv2948c-Rv2950k</td>
<td>Lipid degradation</td>
</tr>
<tr>
<td>Rv3145-Rv3158</td>
<td>nuoA-nuoN (involved in respiration)</td>
</tr>
<tr>
<td>Rv3611c-Rv3616c</td>
<td>AccD4, Pks13, and FadD32 (lipid biosynthesis and degradation)</td>
</tr>
<tr>
<td>Rv3799c-Rv3801c</td>
<td>SodA (Rv3846) and other of unknown functions</td>
</tr>
<tr>
<td>Rv3920c-Rv3932c</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

VOL. 193, 2011 ROLE OF Hsp22.5 IN M. TUBERCULOSIS PATHOGENESIS 3503

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proteomic analyses indicated a potential role for the hsp22.5 gene in the regulation of a large number of genes and operons involved in important pathways for *M. tuberculosis* pathogenesis. The particular role of hsp22.5 in mycobacterial virulence is probably attributed to the long list of downregulated genes which need to be studied in more detail (indirect role). However, proteomic analysis indicated the possible participation of hsp22.5 with other chaperone-encoding genes such as dnaK and grpE in the repression of a large number of genes. Recently, GroEL and to a lesser extent DnaK were found to bind to CD43, a large sialylated glycoprotein found on the surface of hematopoietic cells, that was shown to be necessary for efficient macrophage binding and immunological responsiveness to *M. tuberculosis* (16). Similarly, we suggest the mechanisms involving hsp22.5 in mycobacterial survival under stress are in progress and could strengthen our understanding of the role played by this gene in *M. tuberculosis* virulence during chronic infection.

ACKNOWLEDGMENTS

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REFERENCES


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**TABLE 2. List of mycobacterial proteins downregulated in cultures of the Δhsp22.5 mutant compared to those in the H37Rv strains by use of proteomic analysis**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Probable function</th>
<th>M. tuberculosis H37Rv</th>
<th>Δhsp22.5 mutant</th>
<th>Fold change between Δhsp22.5 mutant and H37Rv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0001</td>
<td>dnaA (replication initiation)</td>
<td>12.6</td>
<td>7.7</td>
<td>−1.6</td>
</tr>
<tr>
<td>Rv0009</td>
<td>cfp22 (protein folding)</td>
<td>28.4</td>
<td>24.8</td>
<td>−1.2</td>
</tr>
<tr>
<td>Rv0129c</td>
<td>ssc (cell wall biosynthesis)</td>
<td>6.6</td>
<td>1.8</td>
<td>−3.7</td>
</tr>
<tr>
<td>Rv0350</td>
<td>DnaK (heat shock protein)</td>
<td>106.4</td>
<td>106.4</td>
<td>−1.0</td>
</tr>
<tr>
<td>Rv0352</td>
<td>GrpE (heat shock protein)</td>
<td>17.7</td>
<td>7.1</td>
<td>−2.5</td>
</tr>
<tr>
<td>Rv0509</td>
<td>hemA (porphyrin biosynthesis)</td>
<td>5.2</td>
<td>1.3</td>
<td>−4.0</td>
</tr>
<tr>
<td>Rv0951</td>
<td>sucC (tricarboxylic acid cycle)</td>
<td>20.8</td>
<td>16.3</td>
<td>−1.3</td>
</tr>
<tr>
<td>Rv1185c</td>
<td>fadD21 (lipid degradation)</td>
<td>13.6</td>
<td>9.9</td>
<td>−1.4</td>
</tr>
<tr>
<td>Rv2196</td>
<td>gcrB (respiration)</td>
<td>7.6</td>
<td>4.8</td>
<td>−1.5</td>
</tr>
<tr>
<td>Rv2245</td>
<td>KasA (fatty acid biosynthesis)</td>
<td>52.9</td>
<td>41.8</td>
<td>−1.3</td>
</tr>
<tr>
<td>Rv2524c</td>
<td>Fas (lipid metabolism)</td>
<td>102.6</td>
<td>66.4</td>
<td>−1.5</td>
</tr>
<tr>
<td>Rv2941c</td>
<td>fadD28 (PDIM biosynthesis)</td>
<td>19.6</td>
<td>12.1</td>
<td>−1.6</td>
</tr>
<tr>
<td>Rv3763</td>
<td>19-kDa lipoprotein (virulence)</td>
<td>17.4</td>
<td>12.5</td>
<td>−1.4</td>
</tr>
<tr>
<td>Rv3825c</td>
<td>pks2 (lipid metabolism)</td>
<td>51.1</td>
<td>40.0</td>
<td>−1.3</td>
</tr>
<tr>
<td>Rv3921c</td>
<td>Possible transmembrane protein</td>
<td>6.4</td>
<td>3.6</td>
<td>−1.8</td>
</tr>
</tbody>
</table>

* Fold change is based on total peptide counts/sample for each protein.