Protective role of *Mycobacterium leprae* small heat-shock protein in heterologous hosts, *Escherichia coli* and *Mycobacterium smegmatis*, grown under stress

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The aim of this study is to examine the *in vivo* role of a small heat-shock protein (sHsp18) from *Mycobacterium leprae* in the survival of heterologous recombinant hosts carrying the gene encoding this protein under different environmental conditions that are normally encountered by *M. leprae* during its infection of the human host. Using an *Escherichia coli* system where *shsp18* expression is controlled by its native promoter, we show that expression of *shsp18* is induced under low oxygen tension, nutrient depletion and oxidative stress, all of which reflect the natural internal environment of the granulomas where the pathogen resides for long periods. We demonstrate the *in vivo* chaperone activity of sHsp18 through its ability to confer survival advantage to recombinant *E. coli* at heat-shock temperatures. Additional evidence for the protective role of sHsp18 was obtained when *Mycobacterium smegmatis* harbouring a copy of *shsp18* was found to multiply better in human macrophages. Furthermore, the autokinase activity of sHsp18 protein demonstrated for what is believed to be the first time in this study implies that some of the functions of sHsp18 might be controlled by the phosphorylation state of this protein. Results from this study suggest that *shsp18* might be one of the factors that facilitate the survival and persistence of *M. leprae* under stress and autophosphorylation of sHsp18 protein could be a mechanism used by this protein to sense changes in the external environment.

INTRODUCTION

*Mycobacterium leprae*, the aetiological agent of leprosy, is known to persist in the host for a long period, and hence poses a major hurdle in eradicating leprosy. The incubation period of *M. leprae* can vary anywhere between 4 and 10 years (Noordeen, 1994). To achieve elimination and eradication of leprosy, sensitive diagnostic methods that can detect the active and persistent bacteria, as well as drugs that could eliminate both these populations, are needed.

Many factors that contribute to the virulence and persistence of *M. leprae* are not well understood. It is important to characterize virulence factors that would advance our understanding on *M. leprae* pathogenesis. One potential virulence factor worth considering is a small heat-shock protein, sHsp18. Young and his co-workers (Young et al., 1985) reported sHsp18 as one of the major immunogenic proteins of *M. leprae*. Studies on the immunological properties of sHsp18 show that this protein is a good stimulator of CD4+ T-cell response (Booth et al., 1988) and is probably involved in inducing protective immunity against *M. leprae* infection (Dockrell et al., 1989). Despite the evidence implying sHsp18 in a protective role and control of infection, there is evidence that suggests that *shsp18* could be an important survival factor for *M. leprae*. Comparative analysis of the genome of *M. leprae* with that of *Mycobacterium tuberculosis* has revealed the loss of a large number of single copy genes, as well as redundant genes, in *M. leprae* (Cole et al., 2001). Interestingly, the *shsp18* gene is retained as a part of the minimal gene set indicating that *shsp18* might have a significant role in the survival, virulence or pathogenesis of *M. leprae*. However, only limited information is available on the role of *shsp18* in this regard. Dellagostin et al. (1995) showed a strong induction of *M. leprae* *shsp18* promoter in recombinant Bacillus Calmette–Gueérin upon entry into macrophages. And, we have demonstrated that SsHsp18 protein acts as a molecular chaperone under *in vitro* conditions (Lini et al., 2008). The present study attempts to characterize the *shsp18* gene and its protein, with particular emphasis on its role in the survival of *M. leprae* under the diverse environments encountered during infection. In this report, we used *Escherichia coli*, a heterologous unrelated...
host and *Mycobacterium smegmatis*, a free-living mycobacterium, as surrogate hosts to examine the functions of *shsp18*. Recombinant *E. coli* allowed us to examine the role of sHsp18 under various stress conditions, while recombinant *M. smegmatis* enabled us to study the role of *shsp18* in infected macrophages. Finally, we demonstrate for the first time that sHsp18 protein is an autokinase, a property that highlights the multifunctional nature of this protein.

**METHODS**

**Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table 1. *M. smegmatis* was grown at 37 °C in M7H9 medium (supplemented with 10% albumin-glucose complex), LB broth or brain heart infusion (BHI) medium containing 0.05% (v/v) Tween-80. Antibiotics (Sigma) were used at the following concentrations: 100 µg ampicillin ml⁻¹; 25 µg kanamycin ml⁻¹; 50 µg apramycin ml⁻¹; 40 µg streptomycin ml⁻¹.

**Thermotolerance experiments.** Fresh overnight culture of M15/pREP4/pH 18 was diluted 100-fold in LB broth with ampicillin and kanamycin, cultured at 37 °C, and IPTG was added to a final concentration of 0.4 mM. After 2 h growth, cultures were shifted to 46, 50 or 53 °C. Samples were taken at 0, 30, 60, 90 and 120 min, serially diluted and plated on LB agar containing antibiotics.

For analysing the protein profile of *E. coli*, cells from 10 ml of culture were centrifuged and the cell pellet, after washing once with 2% (w/v) sarkosyl (N-lauryl-sarkosine), incubated at 25 °C for 20 min and centrifuged at 50 000 r.p.m. for 40 min at 4 °C. The supernatant (outer-membrane fraction) was resuspended in an equal volume of spheroplast buffer [50 mM Tris/HCl (pH 8.0), 18% (v/v) sucrose, 1 mM CaCl₂, 0.5 mM EDTA and 0.5 µg lysozyme ml⁻¹]. After 20 min incubation, cells were spun at 10 000 r.p.m. for 15 min and the supernatant (periplasmic fraction) was recovered. The cell pellet was resuspended in 2 ml TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA] and was sonicated and centrifuged at 10 000 r.p.m. for 4 min at 4 °C. The supernatant was centrifuged at 150 000 g for 40 min at 4 °C. After recovering the supernatant (cytoplasmic fraction), the pellet was resuspended in 1 ml TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA] with 2% (v/v) sarkosyl (N-lauryl-sarkosine), incubated at 25 °C for 20 min and centrifuged at 50 000 r.p.m. for 40 min at 4 °C. The supernatant (inner-membrane fraction) was collected and the pellet (outer-membrane fraction) was resuspended in 200 µl SDS lysis buffer. The proteins in each of the fractions were analysed by SDS-PAGE.

**Stress analysis.** A single colony of XL1/pSET-18Su was transferred to 3 ml LB broth with apramycin and incubated at 37 °C overnight. A 50-fold dilution of the fresh overnight culture was made in medium containing apramycin and the culture was grown till the OD₆₀₀ reached 0.5. Mid-exponential phase cultures of XL1/pSET-18Su were used for exposure to different stresses (Yuan et al., 1996; Cunningham & Spreadbury, 1998) for 1 h, after which the cells were harvested. The harvested cells were resuspended in SDS lysis buffer, diluted to 1 OD₆₀₀ unit of cells per 100 µl and lysed by boiling at 100 °C for 3 min. Equal concentrations of proteins prepared from *E. coli* cells exposed to different stresses were resolved using SDS-PAGE and transferred to nitrocellulose (NC) membrane for Western blotting analysis (Lini et al., 2008). For acid stress, the cells at exponential phase were harvested and resuspended in LB broth at pH 4.0. For oxidative and alcohol stress, H₂O₂ (10 mM) or ethanol (5%) was added to the culture, respectively. To examine the effect of heat and cold shock, the cultures were shifted to 50 or 4 °C, respectively. For stationary phase culture, *E. coli* culture was grown to an OD₆₀₀ of 1.6. Microaerobic conditions were simulated by growing cultures in conical flasks filled with 4/5th volume of medium as opposed to the normal 1/5th volume of medium used for aerobic cultures (Narro et al., 1990). Microaerobic cultures were harvested at both mid-exponential phase (OD₆₀₀=0.5) and stationary phase (OD₆₀₀=1.6).

**Integration of shsp18 in the M. smegmatis genome.** The recombinant plasmid DNA (pSET-18Su) or the vector plasmid (pSET152) was used for electrotransformation. To an aliquot of electro-competent *M. smegmatis* cells, 1 µgDNA was added and the cells were kept on ice for 5 min. This suspension was transferred into an electroporation cuvette (0.1 cm width; Bio-Rad) and electroporated using the following pulse conditions: voltage, 1.4 kV;

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**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
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<td>M15/pREP4</td>
<td>Strain for overexpression of heterologous recombinant proteins</td>
<td>Qiagen</td>
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<tr>
<td>XL1 Blue MRF</td>
<td>Host cloning strain</td>
<td>Stratagene</td>
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<tr>
<td>M. smegmatis</td>
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<tr>
<td>mc₁⁵₅</td>
<td>Wild-type M. smegmatis</td>
<td>Snapper <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>mc₁⁵₅::pSET-18Su</td>
<td>M. smegmatis with an integrated copy of shsp18 from M. leprae</td>
<td>This study</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pQE31</td>
<td>Overexpression vector</td>
<td>Qiagen</td>
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<tr>
<td>pH 18</td>
<td>pQE31 containing the full-length coding sequence of shsp18 (ML1795)</td>
<td>Lini <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>pSET152</td>
<td>Integrative vector</td>
<td>Bierman <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pSET-18Su</td>
<td>pSET152 with full-length shsp18 gene along with 169 bp upstream of the start codon</td>
<td>This study</td>
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resistance, 1000 Ω; capacitance, 25 μF; time, 15 s. The electroporated cells were immediately diluted in 2 ml BHI broth and incubated at 37 °C for 3 h for the expression of antibiotic resistance. The culture was then plated on BHI agar containing apramycin and incubated at 37 °C for 3–5 days.

The plasmid pSET152 is non-replicative in M. smegmatis and its integration into the genome of M. smegmatis was confirmed by a plasmid rescue strategy (Paranthaman & Dharmalingam, 2003). Briefly, genomic DNA isolated from M. smegmatis transformants was digested with BamHI. The digested DNA was purified, self-ligated with T4 DNA ligase and transformed into E. coli XL1 Blue MRF'. Apramycin-resistant E. coli transformants were selected and plasmid DNA was isolated. Restriction analysis of the rescued plasmid was performed to confirm the presence of vector as well as chromosomal DNA.

**Macrophage infection.** A human monocytic cell line, THP-1, was cultured in complete RPMI medium with 10% newborn calf serum supplemented with 10 μg streptomycin ml⁻¹ and 10 U penicillin ml⁻¹. For infection assays, THP-1 cells were grown to 80% confluence and differentiated using 400 ng phorbol-12-myristate-13-acetate ml⁻¹. For infection assays, THP-1 cells were grown to 80% confluence and differentiated using 400 ng phorbol-12-myristate-13-acetate ml⁻¹. Adherent macrophages were harvested and seeded at a concentration of 2.5 x 10⁵ cells ml⁻¹ per well in a 24-well plate with RPMI medium lacking antibiotics. After 24 h, the macrophages were washed and infected with M. smegmatis (opsonized for 1 h with human AB serum) at an m.o.i. of 1. After 2 h, the cells were washed three times with warm Hanks' balanced salt solution (HBSS) and incubated in RPMI medium with 40 μg streptomycin ml⁻¹. At the indicated time points, the medium were removed and after washing three times with warm HBSS, the macrophages were lysed with 1 ml sterile deionized water. The recovered intracellular M. smegmatis was serially diluted and plated on LB agar containing apramycin. The plates were incubated at 37 °C for 3–5 days and the number of c.f.u. obtained was counted. At every time point, intracellular mycobacteria were recovered and the c.f.u. determined from three independent wells. The mean of values from three independent wells was used to calculate the SD and SE. A graph was plotted with the total number of viable intracellular bacteria (y-axis) against time in hours (x-axis). In addition, the percentage of survival of M. smegmatis was plotted against time. Three independent experiments were performed to confirm the observation.

**Autophosphorylation assay.** sHsp18 protein was purified from recombinant E. coli under non-denaturing conditions as per Qiagen's instructions, with minor modifications. All the eluate fractions obtained during purification were checked on SDS-polyacrylamide gel. The fractions showing a single sHsp18 band in SDS-PAGE were pooled and dialysed against the phosphorylation reaction buffer (25 mM Tris/HCl pH 7.0, 1 mM DTT, 1 mM EDTA) at 4 °C for 16 h. The dialysed protein was stored at -20 °C. The autophosphorylation assay for sHsp18 was carried out as described by Premeta et al. (2004). Ten microcurie (3.7 x 10⁶ Bq) of [γ-32P]ATP was added to 5 μg sHsp18 suspended in buffer with 5 mM MgCl₂ and incubated at 30 °C for 60 min. At the end of the reaction, the protein was precipitated with acetone and resolved on a 15% SDS-polyacrylamide gel. The proteins were then transferred to a NC membrane, dried and exposed to X-ray film at -70 °C. The signal was detected by developing the X-ray film using standard procedures.

**RESULTS**

**Effect of heat shock on the survival of E. coli expressing sHsp18**

E. coli M15/pREP4/pH 18 strain that expresses sHsp18 protein (ML1795) upon IPTG induction (Lini et al., 2008) was used to examine the *in vivo* protective effect of sHsp18 during thermal stress. E. coli carrying the vector (pQE31) was used as a control. Cells were grown to mid-exponential phase at 37 °C, and after adding IPTG the culture was grown for another 2 h. At this point, the cultures were shifted to a higher temperature and incubation continued for 2 h. Three different experimental temperatures namely, 46, 50 and 53 °C were used, and the viability of E. coli was assessed by sampling at regular intervals. E. coli exhibited distinct survival kinetics at each of these temperatures (Fig. 1). Irrespective of the presence or absence of sHsp18, E. coli cells were not killed at 46 °C over the 2 h heat-exposure period. However, cultures expressing sHsp18 showed a nearly fourfold increase in cell number at this temperature. At 50 °C, E. coli with or without sHsp18 exhibited a similar pattern of cell survival, and there was no difference in the number of viable cells in cultures carrying the recombinant plasmid with respect to the control. In cultures grown at 53 °C, even though the viability of E. coli was reduced over time, a higher number of viable cells was found in cultures expressing sHsp18. E. coli harbouring sHsp18 exhibited one to two orders of magnitude higher numbers of viable cells when compared to the vector control. These results indicate that sHsp18 conferred a survival advantage on E. coli when grown at high temperatures.

**Fig. 1.** Survival of E. coli overexpressing sHsp18 at different temperatures. E. coli strain M15/pREP4 harbouring pH 18 (overexpressing sHsp18) or pQE31 (vector control) was grown to OD₆₀₀ 0.6 and IPTG was added. After 2 h incubation at 37 °C, cultures were shifted to a higher temperature. At regular intervals, 1 ml culture was serially diluted and plated to determine the number of c.f.u. Considering the number of cells at 0 h of heat shock as 100% survival, the percentage of survival of E. coli was calculated for different time points of exposure to heat shock. Survival of E. coli overexpressing sHsp18 (●) is compared with that of E. coli carrying the empty vector (○) at 46 °C (dashed line), 50 °C (dotted line) and 53 °C (solid line).
Protein profile of recombinant E. coli cells exposed to high temperature

Previously we have reported that sHsp18 functions as a chaperone under \textit{in vitro} conditions (Lini \textit{et al.}, 2008), and the survival advantage for \textit{E. coli} expressing sHsp18 at 46 and 53 °C observed in this study could be attributed to the chaperone function of sHsp18. In order to examine this, the following experiments were carried out. The ability of a heat-shock protein to bind denatured proteins is a prerequisite for its chaperone activity. To demonstrate binding of denatured substrates to sHsp18 under \textit{in vivo} conditions, we analysed the protein profiles of \textit{E. coli} overexpressing sHsp18 grown under heat-shock conditions (Fig. 2). Initially, to determine the effect of thermal stress on cellular proteins, total cell lysate of \textit{E. coli} was prepared under reducing and non-reducing conditions and checked on a polyacrylamide gel. Aggregation of proteins was clearly evident in samples prepared without the addition of DTT from \textit{E. coli} exposed to heat-shock conditions (Fig. 2, lanes 5 and 7). Proteins in the 12K pellet fraction of \textit{E. coli} grown at high temperature could be resolved properly only after DTT treatment (compare lanes 7 and 8, Fig. 2) indicating the presence of protein aggregates, while the proteins in the 12K pellet fraction of \textit{E. coli} grown at 37 °C could be resolved well even without DTT (lane 3 versus lane 4, Fig. 2). Comparison of the DTT-treated soluble and insoluble fractions prepared from \textit{E. coli} cells with and without heat shock revealed a subset of proteins that were present in the 12K supernatant before heat shock but appeared only in the 12K pellet after heat shock (indicated by black triangles in Fig. 2). These proteins represent the \textit{E. coli} proteins, which are sensitive to high temperature. A set of proteins were induced on heat shock (indicated by black circles in Fig. 2). Next, to determine the effect of heat shock on sHsp18 per se, a time-course analysis during heat shock was performed, and the protein profile of the soluble and insoluble fractions of \textit{E. coli} collected at different time points were examined (Fig. 3). As the duration of heat shock increased, the level of sHsp18 decreased in the soluble fraction (Fig. 3a) with a concomitant increase in the pellet fraction (Fig. 3b). We have reported previously the appearance of a truncated form of sHsp18 (16.7 kDa) when \textit{E. coli} cells overexpressing sHsp18 were lysed (Lini \textit{et al.}, 2008). The data in Fig. 3 show that the level of this truncated protein in both the soluble and insoluble fractions decreased with the increasing duration of heat shock, and only negligible levels were detected by 120 min of heat shock.

Native promoter driven expression of \textit{M. leprae} \textit{shsp18} \textit{in E. coli}

Recombinant plasmid pSET-18Su, which harbours the \textit{shsp18} sequence along with its native promoter region (ORF and 168 bp upstream of the start codon), was constructed and introduced into \textit{E. coli} XL1 Blue MRF’. Expression of sHsp18 protein was checked in recombinant
E. coli that was grown to mid-exponential phase. Total cell protein was separated by SDS-PAGE (Fig. S1a, available in JMM Online), transferred to NC membrane and probed with anti-sHsp18 antibodies (Fig. S1b). The signal for a protein corresponding to ~17 kDa was detected and this mass correlates with the deduced molecular mass of 16.7 kDa for sHsp18. To determine the location of sHsp18, proteins from different cellular compartments of E. coli were probed with anti-sHsp18 antibodies and sHsp18 was detected exclusively in the periplasmic fraction (Fig. S1c).

Expression of sHsp18 under stress conditions

M. leprae, during its infection process, encounters different stress conditions such as variations in temperature, low oxygen tension, low pH, exposure to organic compounds, ROS (reactive oxygen species), RNS (reactive nitrogen species), nutrient limitation etc. Since M. leprae is uncultivable, we used E. coli as the surrogate host to examine the response of the shsp18 promoter to some of the stress conditions mentioned above. E. coli harbouring the shsp18 gene with its upstream promoter region was grown to mid-exponential phase and exposed to the indicated stress conditions. Stationary phase represents a nutrient-depleted stress condition, and the response to this stress was analysed by growing E. coli to OD$_{600}$ 1.6 (Ren et al., 2004). To study the effect of hypoxia, E. coli was grown under microaerobic condition and harvested at both the exponential (OD$_{600}$ =0.5) and stationary phase (OD$_{600}$ =1.6) of growth. Total cell lysates were prepared from E. coli cells exposed to each of these stress conditions, resolved on a SDS-PAGE gel and after transfer to NC membrane was probed using anti-sHsp18 antibodies (Fig. S2a). The expression level of sHsp18 varied across the different conditions and to quantify the level of sHsp18 protein expression, the immunoblot was analysed using ImageQuant software (Fig. S2b). The expression level of sHsp18 in the aerobic mid-exponential phase culture was considered as the basal value to calculate the fold change in expression of sHsp18 under a specific stress condition (Table 2). The highest expression of sHsp18 was detected in cells grown to stationary phase under microaerobic conditions. In addition, significant induction of sHsp18 was observed in microaerobically grown exponential phase E. coli cells as well as in aerobically grown stationary phase cells. Induction was lower but significant in cells subjected to oxidative stress, heat shock and ethanol stress. Although acid stress reflects the acidic environment within the phagolysosomes of macrophages, the shsp18 promoter did not respond to this stress.

Integrative cloning of shsp18 in the M. smegmatis genome by site-specific integration

To analyse the function of shsp18 in a mycobacterial host, a copy of shsp18 gene along with its promoter was introduced into the genome of M. smegmatis mc$	extsuperscript{2}$155. The recombinant plasmid, pSET-185u, was electrotransformed into M. smegmatis and interaction of the attB (in the pSET152 vector) with the attB site (in the M. smegmatis genome) leading to homologous recombination resulted in apramycin-resistant transformants. Integration of the plasmid in these transformants (mc$	extsuperscript{2}$155::18Su) was further confirmed by a plasmid rescue strategy (Fig. S3) (Paranthaman & Dharmalingam, 2003). The empty vector, pSET152 was integrated similarly in M. smegmatis (mc$	extsuperscript{2}$155::PSET152) and was used as a control. We have shown earlier (Santhosh et al., 2005) that integration of pSET152 occurs at a unique site in the genome of M. smegmatis, resulting in a single copy of the gene of interest integrated.

Role of shsp18 in the survival of M. smegmatis in macrophages

M. leprae has been shown to survive and multiply inside the host macrophages (Frehel & Rastogi, 1987; Mor, 1983; van der Wel et al., 2007). To assess the role of shsp18 in the survival of mycobacteria inside these phagocytic cells, a bacterial killing assay was performed. THP-1-derived macrophage cells were prepared as described in Methods and infected with M. smegmatis at an m.o.i. of one, and the survival of the mycobacteria inside the macrophages was assessed over a period of 3 to 120 h post-infection. At specific time points, the macrophages were lysed and the number of released viable bacteria was determined. In comparison to the control strain, no significant difference was observed in the survival of recombinant bacteria carrying shsp18 up to 36 h of infection (Fig. 4a, b). The

Table 2. Changes in the expression level of sHsp18 across different stress conditions

sHsp18 levels in the immunoblot (Fig. S2a) were quantified using ImageQuant software (GE Healthcare). The expression level of sHsp18 in the aerobic mid-exponential phase culture was considered as the reference level to determine the fold change in the expression of sHsp18 in cells exposed to different stress conditions.

<table>
<thead>
<tr>
<th>Stress stimuli</th>
<th>Aerobic stationary</th>
<th>Microaerobic</th>
<th>Acid</th>
<th>Oxidative</th>
<th>Ethanol</th>
<th>Heat</th>
<th>Cold</th>
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<td></td>
<td></td>
<td>Mid-exponential</td>
<td></td>
<td>Stationary</td>
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<tr>
<td>Fold change*</td>
<td>1.8</td>
<td>1.4</td>
<td>2.4</td>
<td>0.8</td>
<td>1.3</td>
<td>1.2</td>
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</table>

*Fold change=sHsp18 level in stress-exposed culture/sHsp18 level in aerobic mid-exponential phase culture.
survival pattern of *M. smegmatis* during an extended period of infection indicated that the bacteria were killed gradually until 48 h, followed by a sudden but transient increase in cell number until 72 h. The cell survival declined again after 72 h. During the 48–72 h post-infection period, mc2155::18Su exhibited a prominent difference in survival pattern (Fig. 4c). A 3.14-fold increase in the survival percentage (from 14 to 58%) was observed for *M. smegmatis* harbouring a copy of *shsp18*, whereas this percentage increased only by 1.09-fold in the case of the control strain (11–23%) (Fig. 4d). These results were confirmed by repeating the experiment three times. These findings suggest that *shsp18* facilitates the multiplication of *M. smegmatis* within macrophages during the regrowth stage of infection.

**Autophosphorylation of sHsp18**

The recombinant protein purified from *E. coli* under non-denaturing conditions (Fig. 5a) was used for this experiment. Phosphorylation of purified sHsp18 was carried out at 30 °C for 60 min in the presence of Mg2+ ions with radiolabelled ATP as the phosphate donor. The protein was separated on a SDS-polyacrylamide gel, transferred to NC membrane, and then the membrane was exposed to X-ray film. An intense signal was detected in the autoradiogram at a position corresponding to that of sHsp18 (Fig. 5b). On probing the NC membrane with anti-sHsp18 antibodies, it was confirmed that the phosphorylation signal was from sHsp18 protein (Fig. 5c), clearly indicating that sHsp18 is capable of phosphorylating itself.

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**Fig. 4.** Role of *shsp18* in the survival of *M. smegmatis* in macrophages. Phorbol-12-myristate-13-acetate differentiated THP-1 cells were infected with *M. smegmatis* mc2155::pSET152 (black diamonds) or mc2155::pSET-18Su (black triangles) at an m.o.i. of 1 and the infection was allowed to proceed for 2 h. After removing the unphagocytosed as well as surface-bound mycobacteria, the infected macrophages were maintained under suitable conditions as described in Methods for 3–120 h. At specific time points, macrophages were lysed and the number of mycobacteria surviving within the macrophages was determined by serial dilution and plating. The c.f.u. values are mean values derived from three independent wells at the indicated time points. Survival of *M. smegmatis* in the macrophages was analysed over different infection periods of 3–6 h (a), 3–36 h (b) and 3–120 h (c). *M. smegmatis* harbouring an integrated copy of the *shsp18* gene (white bars) exhibits a fourfold higher survival over the control (black bars) by 72 h after infection, which is clearly evident when the intracellular survival of *M. smegmatis* is plotted against infection time (d).
DISCUSSION

Conventional approaches for studying the functions of a gene, such as gene knockout, silencing and complementation, are not applicable to M. leprae, since this pathogen is uncultivable. Therefore, we used surrogate hosts, E. coli (Basu et al., 1996) and M. smegmatis (Santhosh et al., 2005) to understand the role of the shsp18 gene in the survival and persistence of M. leprae in the human host. Both these systems were used to analyse the functions of the cloned shsp18 under experimental conditions that mimic the environment encountered by M. leprae in the macrophages and granulomas. The expression of shsp18 in recombinant E. coli was significantly increased in response to low oxygen tension, nutrient limitation and oxidative stress, while to a lesser extent under heat, ethanol and cold shock. These stress conditions generally perturb the integrity of cellular proteins and chaperone proteins are required to prevent their denaturation and aggregation, implying the involvement of the in vivo chaperone activity of shsp18 in the prevention of such stress-induced denaturation of proteins. Furthermore, the enhanced multiplication of recombinant M. smegmatis harbouring the shsp18 gene in the macrophages indicates a role for shsp18 in the prevention of macrophage-induced damage. Autophosphorylation of shsp18 protein implies that this protein might have additional unknown functions.

Previously, we have shown the in vitro molecular chaperone activity of shsp18 (Lini et al., 2008). In this study, we demonstrated the in vivo chaperone activity of this protein through its ability to confer thermotolerance to recombinant E. coli. The multiplication of E. coli at 46 °C and an enhanced survival at 53 °C could be correlated with the presence of high levels of shsp18. An increase in cell number observed for E. coli harbouring shsp18 at 46 °C suggests that in addition to the protection provided by the major heat-shock proteins, prior induction of shsp18 offers additional protection and stabilization that promotes the growth of E. coli. However, at 53 °C, the presence of shsp18 could be associated with maintenance of cell viability. At this lethal temperature, when E. coli is killed rapidly even before it could evoke a heat-shock response, the pre-existing shsp18 is essential for the rescue of E. coli cells.

Association between shsp18 and temperature-sensitive cellular proteins during thermal stress might be responsible for the enhanced survival of E. coli. We showed that shsp18, predominantly a soluble protein even at higher temperatures, was recovered in the insoluble protein fraction (12K pellet) of E. coli cells exposed to high temperature. A plausible explanation for this observation is that upon exposure to elevated temperature, shsp18, by virtue of its chaperone activity, binds to the heat-sensitive E. coli proteins to maintain them in a folding-competent state preventing their irreversible aggregation (Lee & Vierling, 2000). At early time points of heat shock, this shsp18-protein complex might be soluble. On prolonged exposure to higher temperature, with an increase in the ratio of denatured proteins bound to shsp18, the complex becomes insoluble, resulting in an increase in the concentration of shsp18 in the pellet fraction. Similar to shsp18, LbpA and LbpB have also been shown to co-sediment with intracellular heat-induced aggregated proteins (Laskowska et al., 1996).

During cell lysis of E. coli overexpressing shsp18, a fraction of shsp18 undergoes cleavage resulting in a truncated protein with a mass of 16.7 kDa in addition to the 19.3 kDa full-length his-tagged protein (Lini et al., 2008). In this study, we observed a decrease in the level of this 16.7 kDa form in cells exposed to higher temperature with a concomitant increase in the 19.3 kDa form of shsp18. In heat-stressed cells, when temperature-sensitive E. coli proteins are bound to shsp18 to form a complex, the cleavage site that generates the 16.7 kDa protein is probably masked resulting in a decrease in shsp18 truncation. This is further supported by the observation that only negligible levels of truncated shsp18 were detected in cells exposed to heat shock for 120 min. This could be due to an increase in the amount of denatured proteins bound to shsp18, thereby masking the cleavage site in all shsp18 molecules. Thus, aggregation of shsp18...
and the associated decrease in its truncation imply the binding of sHsp18 to denatured protein substrates in vivo. Generally, α-crystallin-like small heat-shock proteins are expressed at basal levels and are highly induced only under some specific stress conditions (Narberhaus, 2002). We found that oxygen deprivation or hypoxia influences the expression of shsp18 and this can be correlated with the lifestyle of M. leprae. From earlier reports (Cosma et al., 2003), it is clear that M. leprae seems to be well adapted for survival under microaerobic conditions and our results suggest that sHsp18 might be one of the factors essential for adaptation of M. leprae to hypoxic conditions. Furthermore, sHsp18 protein was found to accumulate in stationary phase culture, which represents a nutrient-depleted state (Navarro Llorens et al., 2010), indicating that transition to stationary phase is yet another cue for increased expression of shsp18. Interestingly, the highest level of shsp18 induction was observed under a combination of signals, hypoxia and stationary phase, both of which are characteristic of the environment within the granulomas where mycobacterial pathogens persists in a dormant state for extended periods (Cunningham & Spreadbury, 1998). The presence of higher levels of sHsp18 in the stationary phase culture may also be the result of a low rate of turnover of this protein. sHsp18 was shown to be located in the periplasm and outer membrane of E. coli (Lini et al., 2008) and in the cell wall and membrane compartments of M. leprae (Marques et al., 2004). These data indicate that this protein could also have a role in cell wall stabilization. sHsp18 is capable of protecting and stabilizing the components of the protein translational machinery in E. coli during stress (our unpublished data). Taken together, these data suggest that sHsp18 might be involved in protecting the essential components of the pathogen to maintain the cell viability during unfavourable conditions. Experiments designed to examine the role of shsp18 in M. smegmatis further support this.

Data presented above show that hypoxia, nutrient depletion and oxidative stress could induce the expression of shsp18. Since M. leprae encounters such environments within the macrophages, the role of shsp18 in the survival of mycobacteria during infection of macrophages was assessed. For these studies, M. smegmatis was chosen as the surrogate host and a single copy of shsp18 gene was introduced into its genome. We observed a specific pattern in the survival of M. smegmatis within the macrophages similar to that reported by others (Anes et al., 2006; Jordao et al., 2008), which involved four distinct phases: a rapid killing phase followed by an intracellular growth phase, a second killing phase and a final slow killing phase. During the intracellular growth phase, M. smegmatis cells harbouring shsp18 complete two rounds of replication as against only one for M. smegmatis without shsp18. This suggests that when the environment inside the macrophages is favourable for growth, M. smegmatis with shsp18 recovers quickly to multiply faster. The ability of shsp18 to facilitate mycobacterial growth within macrophages supports our earlier data that the chaperone activity of sHsp18 might have a role in maintaining cell viability under unfavourable conditions.

Based on the diverse nature of signals to which shsp18 responds, it is plausible that sHsp18 protein has additional functions. α-Crystallin, the representative protein of the family of sHsps, was shown to autophosphorylate (Kantorow & Piatigorsky, 1994), which has been implicated in the regulation of expression of other genes (Pietrowski & Graw, 1997). Our experiments show that sHsp18 of M. leprae is capable of phosphorylating itself. Autokinase activity has been demonstrated only for few α-sHsps, such as human and bovine αA and αB crystallin (Kantorow & Piatigorsky, 1994), HspX of M. tuberculosis (Preneta et al., 2004) and rat Hsp27 (Chowdary et al., 2004), and this is, to the best of our knowledge, the first time autophosphorylation of M. leprae sHsp18 has been demonstrated. Since many kinases that autophosphorylate can also phosphorylate other protein molecules, it is reasonable to expect that the autokinase activity of sHsp18 need not necessarily be restricted to itself but might be extended to the phosphorylation of other proteins as well. This property gains significance particularly in view of the distribution of sHsp18 in the cell wall, as well as membrane compartments, of M. leprae (Marques et al., 2004). It is plausible that the phosphorylation of sHsp18 could be a mechanism to sense changes in the external environment in order to modulate its own cellular signalling system enabling M. leprae to adapt to the changing environments. Since protein kinases are generally the key regulators of cellular signalling, identifying the targets of sHsp18 phosphorylation and mapping the signalling network would improve our understanding on the role of sHsp18 in pathogenesis.

Conclusions

This study has revealed hitherto unexplored functional facets of sHsp18. The diversity in signals that induce shsp18 and the multiplicity of functions of sHsp18 protein indicates that shsp18 is indeed indispensable for the survival of M. leprae. Adaptation of M. leprae to any stressful environment would involve a consortium of genes and proteins resulting in a coordinated stress response. Autokinase property of sHsp18 indicates a possible mechanism, however identification of these additional proteins which network with SHsp18 to promote the pathogenesis of M. leprae is essential to design effective vaccination strategies.

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