Stress induced alteration of \textit{shsp18} expression in \textit{E. coli}

INTRODUCTION

\textit{M. leprae} shows remarkable ability to survive as well as persist over long periods in the host cells (Cosma \textit{et al.}, 2003). Viable bacteria have been demonstrated in leprosy patients at different stages of the disease and treatment. Studies from our lab have shown the expression of \textit{M. leprae} genes (\textit{shsp18} and \textit{mce} operon genes) in the biopsies of patients across the leprosy spectrum starting from the earliest stage, namely TT. This indicates the presence of metabolically viable bacteria across the leprosy spectrum (Shabaana \textit{et al.}, 2003; Santhosh \textit{et al.}, 2005; Lini \textit{et al.}, 2009). Significant amounts of intact bacilli have been demonstrated in the nerve and skin biopsies from multibacillary leprosy patients who had been treated with antibiotics as per the WHO regimen (Shetty \textit{et al.}, 1992). The viability of bacteria has been detected in 10-15\% of the multibacillary leprosy patients even after two years of multidrug therapy (Katoch \textit{et al.}, 1989). Our own studies using biopsy material of LL patients also showed that mRNA of bacterial \textit{shsp18} gene could be detected even after two years of MDT (Lini \textit{et al.}, 2009). Even the bone marrow of lepromatous leprosy patients has been indicated to be a reservoir of viable \textit{M. leprae} (Suster \textit{et al.}, 1989). The occurrence of sub-clinical infection in up to 5\% of healthy individuals has been shown in Indian (Ramaprasad \textit{et al.}, 1997) and Indonesian population (Hatta \textit{et al.}, 1995) and presence of such infection reinforces the fact on persistence of \textit{M. leprae}.

\textit{M. leprae} invading the human host has three main targets - peripheral neural tissues (Schwann cells), small vessels (endothelial cells and pericytes) and the monocyte-macrophage system (Abulafia and Vignale, 1999). The route of infection of \textit{M. leprae} is illustrated in figure 4.1. The principal means of transmission of \textit{M. leprae} is probably through the spread of nasal secretions through aerosols and subsequent uptake through nasal or respiratory mucosa (Noordeen, 1994). However, other routes such as through broken skin (Leiker, 1977) and penetrating wounds have not been ruled out (Hastings \textit{et al.}, 1988). The pathogen enters the lymph and blood.
Figure 4.1: Schematic representation on the routes of infection of *M. leprae*.
vessels to reach the nerves and its Schwann cells, where it can multiply, disseminate, begin new invasive cycles and form perineural granulomas. From the site of infection and also en route to the Schwann cells, the bacilli may be phagocytosed by macrophages. These macrophages may further develop into epitheloid granuloma (in tuberculoid leprosy) or lepromatous granuloma (in lepromatous leprosy) (Abulafia and Vignale, 1999). Such granulomas formed in response to persistent intracellular pathogens are known to be the reservoirs of viable organisms (Cosma et al., 2003).

Macrophages are the primary host cells for *M. leprae* where it encounters the aggressive host bactericidal mechanisms such as low pH (Hackam et al., 1997), reactive oxygen (Babior, 2000) and nitrogen intermediates (Nathan and Shiloh, 2000), which have to be neutralized for its survival and establishment of an infection. In addition, persistence is a hallmark of pathogens such as *M. leprae* and *M. tuberculosis* (Cosma et al., 2003). During such long term infection, *M. leprae* residing in granulomas encounters a dynamic range of hostile environments like the highly oxidative phagocytic compartment of macrophages, the potentially hypoxic and acidic milieu of granulomas. Hence, the ability of *M. leprae* to establish an active or latent disease relies to some extent on the powerful mechanisms it has evolved to parasitize the host cells. It adopts multiple strategies to reduce the overall exposure to anti-bacterial defenses of the host cells and also to neutralize their effectiveness. Such strategies include inhibition of phagosome-lysosome fusion in macrophages (Frehel and Rastogi, 1987) and Schwann cells (Alves et al., 2004), surviving the unfavorable conditions by taking advantage of the components of its complex cell wall (Chan et al., 1991; Guenin-Mace et al., 2009), formation of electron-transparent zones (Sibley et al., 1987), expression of resistance mechanisms against reactive oxygen species (Neill and Klebanoff, 1988) and nitrogen intermediates (Fabozzi et al., 2006). In addition to these, various other cellular factors might also be involved in such adaptive mechanisms that enable the survival of the pathogen in the hostile environments of the host.

One family of proteins that has been implicated in resistance against stress conditions is the α-crystallin type of small heat shock proteins (de Jong et al., 1993; Stewart et al., 2005). A classic example of this, are the *shsps* (*hspX* and *acr2*) of *M. tuberculosis*. These small heat shock protein genes have been shown to be inducible in response to multiple stress conditions including hypoxia (Sherman et al.,
2001), nitric oxide (Voskuil et al., 2003), heat shock (Stewart et al., 2002), oxidative stress (Schnappinger et al., 2003) and stationary phase growth (Cunningham and Spreadbury, 1998). Similarly, the shsp of another intracellular pathogen, Legionella pneumophila, is also induced during intracellular infection, heat shock, oxidative stress and osmotic shock (Abu Kwaik and Engleberg, 1994).

*M. leprae* encoded sHsp18, described earlier is also an α-crystallin like small heat shock protein. Dellagostin and co-workers (1995) have shown that *shsp18* gene can be induced intracellularly in the macrophages. Apart from this, the function of this gene or protein in the context of stress has not been studied so far. Hence, the objective of this part of the study was to characterize the response of *shsp18* gene to diverse stress conditions using *E. coli* as the surrogate host. Surrogate hosts have played an important role in developing our knowledge of mycobacterial genetics by allowing the functional analysis of genes and regulatory sequences particularly for the slow growing or non-cultivable pathogenic *Mycobacterium*. And, *E. coli* has been widely used as surrogate cloning hosts (Clark-Curtiss et al., 1985) which when combined with the conventional recombinant protein technology has enabled the immunological and biochemical studies of various cellular components of the pathogenic mycobacteria. In the present study, in order to analyze the stress response of *shsp18*, this gene was cloned along with its native promoter and transformed into *E. coli*. The expression and localization of the protein was examined in *E. coli*. Recombinant *E. coli* carrying this *shsp18* was subjected to different stress conditions, namely acid, peroxide, ethanol, heat and cold shock and the induction of *shsp18* in response to each of these stresses was analyzed.

**RESULTS**

**Cloning of shsp18 with upstream promoter sequence**

*shsp18* was cloned with its upstream region in pSET152 following the strategy shown in figure 4.2. From *M. leprae* infected armadillo spleen tissue, the bacteria were isolated and genomic DNA was prepared as described in materials and methods. This genomic DNA was used as the template and *shsp18* gene with its upstream region (*shsp18Su*) was amplified by PCR using *Pfu* polymerase. The upstream region in the PCR amplicon included the 168 nucleotides ahead of the ATG start codon which was shown to carry the promoter region (Dellagostin et al., 1995).
Figure 4.2: Cloning of shsp18Su in pSET152 vector. 

A. Sequence of shsp18 gene (447 bp) with the upstream region (168 bp-underlined). Position of the forward and reverse primers used for amplifying the entire 615 bp region is indicated in red italics. The transcriptional start site demonstrated by Dellagostin et al., (1995) is shown with an *. 

B. Schematic representation of the strategy used for cloning of shsp18Su. # indicates the upstream promoter region of shsp18.
In this study, pSET152 vector was used for cloning *shsp18Su*. pSET152 is an integrative vector (Wilkinson *et al.*, 2002) derived from the site-specific recombination system of the *Streptomyces* temperate phage φC31. This cloning vector carries an *E. coli* origin of replication and hence can be propagated as plasmid in *E. coli*. The pSET152 and other integrating vectors based on the φC31 recombination system are used widely in *Streptomyces* research. These vectors are non-replicative in *Streptomyces* but can integrate into the chromosomal attachment site of bacteriophage φC31 (Biermann *et al.*, 1992). An apramycin gene serves as the selection marker. Although pSET152 is a *Streptomyces* vector, its integration in *M. smegmatis* (Santhosh *et al.*, 2005) and *Rodococcus equi* (Hong and Hondalus, 2008) has been demonstrated. In the present study, pSET152 was chosen for cloning *shsp18* gene so as to integrate it into the *M. smegmatis* genome. The presence of such a stable single copy of *shsp18* is essential to simulate the environment found in *M. leprae*. Therefore, the PCR amplicon, *shsp18Su* was cloned in the *Eco*RV site of pSET152 and the recombinant plasmid pSET-18Su was transformed in *E. coli*, XL1 Blue MRF’. Transformants were then selected on apramycin plates.

Plasmid DNA from two of the transformants, T1 and T2, was isolated and the presence of *shsp18* was confirmed by restriction analysis as shown in figure 4.3. When the recombinant plasmids and pSET152 were digested with *BamHI* (a site present only in the vector), all three plasmids were linearized as shown in lanes 2, 5 and 8. Unlike *BamHI*, *BglII* site is present only in *shsp18* and linearization with this enzyme indicates the presence of *shsp18*. When the three plasmids were digested with *BglII*, only pSET-18Su from T1 and T2 linearized (lanes 6 & 9) while pSET152 (lane 3) was not cleaved. This restriction analysis thus confirms the presence of *shsp18* gene in the plasmids from both the transformants, T1 and T2.

Although both the transformants harbored the cloned *shsp18Su* in pSET152 vector backbone, only one of the transformants (T1) was used for all studies carried out subsequently.

**Single nucleotide polymorphism in *shsp18Su***

Shabaana and co-workers (2003) have shown that the *shsp18* gene exhibits a single nucleotide polymorphism at the 154th base position (T => C) and because of this SNP, the 52nd amino acid in sHsp18 is either a serine (‘S’ type) or proline (“P” type).
Figure 4.3: Restriction analysis of pSET-18Su. Plasmid from two transformants, T1 and T2, were digested with BamHI and BglII and electrophoresed on a 0.7% agarose gel. Lane 1, pSET152/uncut; 2, pSET152/BamHI; 3, pSE152/BglII; 4, pSET-18Su (T1)/uncut; 5, pSET-18Su (T1)/BamHI; 6, pSET-18Su (T1)/BglII; 7, pSET-18Su (T2)/uncut; 8, pSET-18Su (T2)/BamHI; 9, pSET-18Su (T2)/BglII; 10, λ HindIII/EcoRI DNA marker.
These authors have also described a simple way of identifying this SNP based on Sau3A1 restriction pattern which is illustrated in figure 4.4A. Digestion of shsp18 of ‘S’ type with Sau3A1 generates a 388 bp fragment along with four fragments of size less than 100 bp. In the ‘P’ type, the SNP generates an additional Sau3A1 restriction site. This new site will result in two fragments of sizes 319 bp and 69 bp instead of the 388 bp DNA fragment obtained in the ‘S’ type. Thus, Sau3A1 restriction generates distinct DNA band pattern depending on the SNP present.

The source of shsp18 for the present study was the genomic DNA of M. leprae from infected armadillo. To determine the sequence at the SNP position of this gene, shsp18 was amplified by PCR using pSET-18Su as the template and digested with Sau3A1 as shown in figure 4.4B. The size of the largest fragment obtained on Sau3A1 digestion was greater than 350 bp, which is the one expected (388 bp) if shsp18 is of ‘S’ type. Thus, shsp18 of armadillo derived M. leprae was confirmed as the ‘S’ type as reported earlier (Booth et al., 1988; Shabaana et al., 2003).

**Expression of sHsp18 in E. coli**

It was essential to determine whether the E. coli system was capable of recognizing the mycobacterial promoter and express shsp18. The gene expression was confirmed by presence of sHsp18 protein in the total cell lysate of the recombinant E. coli, XL1/pSET-18Su. XL1/pSET152 served as the vector control for the studies carried in this part of the study.

Protein profile of the total cell lysate of recombinant E. coli is shown in figure 4.5A. No difference could be observed between the two profiles. However, when these samples were probed with anti-sHsp18 antibody, sHsp18 was detected in the XL1/pSET-18Su lysate (lane 3 of figure 4.5B). The signal obtained for sHsp18 corresponds to an approximate molecular mass of 17 kDa which correlates well with the calculated molecular mass of 16.7 kDa for the protein.

**Localization of sHsp18 in E. coli**

The subcellular localization of sHsp18 was analyzed after fractionating the proteins of different compartments of E. coli. Proteins of the subcellular fractions, namely, the periplasm, cytoplasm, outer membrane and inner membrane, were separated on a
Figure 4.4: Single Nucleotide Polymorphism of the cloned *shsp18Su*. A. Illustration on the use of *Sau3A1* restriction analysis to determine the SNP in the *shsp18* gene. The common *Sau3A1* sites of both ‘P’ and ‘S’ types are shown in blue arrows. Red arrow represents the additional *Sau3A1* site generated by the SNP (shown in red font) in the ‘P’ type. Size of the DNA fragments obtained by *Sau3A1* digestion is given for both types. B. Restriction analysis of *shsp18Su*. Lane 1, *shsp18Su* PCR amplicon; 2, *shsp18Su/Sau3A1*; 3, 50 bp DNA ladder.
Figure 4.5: Analysis of *shsp18* expression in *E. coli*. A. *E. coli* carrying the vector plasmid pSET152 or pSET-18Su, was grown and the cell lysate was prepared as in materials and methods. Twenty microgram of the total cell protein was separated on a 12% SDS-polyacrylamide gel and visualized with colloidal coomassie stain. Lanes 1, molecular weight marker; 2, XL1/pSET152; 3, XL1/pSET-18Su. B. Immunoblot of *E. coli* total cell lysate. *E. coli* samples shown in A were transferred on to a NC membrane. The membrane was probed with anti-sHsp18 antibody and the blot was developed. Lane details are the same as that in A. Dotted arrow indicates the position of sHsp18.
12% SDS-polyacrylamide gel (figure 4.6A) and was analyzed by western blotting also (figure 4.6B).

In the immuno blot of subcellular fractions of *E. coli*, sHsp18 was detected only in the periplasmic fraction from XL1/pSET-18Su (lane 5 of figure 4.6B). Purified His-tag sHsp18 was included as the positive control (lane 9) in which both 19.3 kDa and 16.7 kDa forms were observed. Molecular mass of sHsp18 detected in the periplasmic fraction (lane 5) matches in size with the lower 16.7 kDa form of the purified sHsp18 (lane 9).

Detection of sHsp18 clearly indicates that the mycobacterial promoter was recognized by the *E. coli* system and hence, *shsp18* was transcribed and translated. Further, the expressed protein was also found to localize in the periplasm of *E. coli*.

**Response of *shsp18* to different stress stimuli**

Recombinant *E. coli* carrying *shsp18Su* was used for analyzing the stress response of this gene. sHsp18 protein level was used as a measure of *shsp18* induction in response to a stress. For this analysis, XL1/pSET-18Su was grown under identical conditions till the cultures reached the mid-log phase. At this point, each culture was subjected to one of the following stress conditions – acid stress at pH 4.0, oxidative stress with 10 mM H$_2$O$_2$, alcohol stress with 5% ethanol, heat shock at 50 °C or cold shock at 4 °C. The cultures were maintained in the respective conditions for 30 minutes and harvested. For stationary phase culture, *E. coli* was incubated until the culture reached an OD of 1.6. In addition, XL1/pSET-18Su cultures were grown under microaerobic conditions as described in materials and methods. This culture was harvested at the mid-log phase as well as in the stationary phase ($A_{600}=1.6$) and included along with the above samples for analysis. The normal aerobic mid-log phase culture served as the reference for comparison.

Total cell lysate of *E. coli* was analyzed on SDS-polyacrylamide gel as shown in figure 4.7A. Since there was no difference in the protein profile, expression of sHsp18 was determined by western analysis. The result of this analysis is shown in figure 4.7B.
Figure 4.6: Subcellular localization of sHsp18 in *E. coli*. A. *E. coli* cells were cultured and fractionated as described in materials and methods. Proteins (5 µg) of different cellular fractions from XL1/pSET152 (lanes 1-4) and XL1/pSET-18Su (lanes 5-8) were separated on a 12% SDS-polyacrylamide gel. Lanes 1 & 5, periplasmic fraction; 2 & 6, cytoplasmic fraction; 3 & 7, outer membrane fraction; 4 & 8, inner membrane fraction; 9, purified sHsp18. B. Immunoblot analysis of the subcellular protein fractions. Protein samples shown in A were taken for immunoblot analysis with anti-sHsp18 antibody. A portion of the immunoblot corresponding to the sHsp18 regions is shown. Details of lanes are the same as that in the gel shown in A. Solid arrow in the blot indicates the position of sHsp18.
Figure 4.7: Expression of sHsp18 in *E. coli* exposed to stress. A. Protein profile of *E. coli* exposed to different stress conditions. XL1/pSET-18Su was grown and subjected to different stress as described in materials and methods. One OD equivalent of cells were lysed in 100 µl of SDS-lysis buffer and 10 µl of the cell lysate (corresponding to 20 µg) was analyzed on a SDS-polyacrylamide gel. Lanes represent the total cell lysate of *E. coli* maintained at the following conditions. Lane 1, aerobic culture at mid-log phase; 2, aerobic culture at stationary phase; 3, microaerobic culture at mid-log phase; 4, microaerobic culture at stationary phase; 5, acid stress; 6, oxidative stress; 7, ethanol stress; 8, heat shock; 9, cold shock; 10, purified sHsp18; 11, molecular weight marker. B. Western blot analysis. *E. coli* samples shown in A were transferred to a NC membrane, probed with anti-sHsp18 antibody and the blot developed as described in materials and methods. Portion of the blot corresponding to the sHsp18 region is shown. Solid arrow indicates the position of 16.7 kDa sHsp18 produced in response to stress. Dotted arrow indicates the position of the purified His-tag sHsp18 (19.3 kDa).
A visual inspection of this western blot reveals that amongst the stress conditions analyzed, *shsp18* induction is very prominent and significant in the stationary phase culture (in both aerobic and microaerobic growth) as well as in the mid-log phase culture of *E. coli* grown under microaerobic conditions. Although low to moderate level of induction was observed in other stress conditions, it was important to quantify these levels to confirm this observation. Hence, the blot (shown in figure 4.7B) was taken for ImageQuant™ analysis as described in materials and methods and SHsp18 levels were quantified as band volume (figure 4.8). The band volume of SHsp18 of the mid-log phase aerobic culture was used as the basal value to calculate the fold increase in SHsp18 expression in the stress conditions analyzed. These results are given in table 4.1.

A fold change of greater than one was considered as induction of *shsp18*. Greater the fold change, higher the induction in that particular stress condition. From the figure 4.8 and table 4.1, it is apparent that *shsp18* induction occurs under all stress conditions analyzed except acid stress. However, stationary phase and microaerobiosis are the conditions which strongly influence *shsp18* expression. And, higher levels of SHsp18 expression were observed with a combination of these two stimuli, that is, the stationary phase culture under microaerobic conditions.

**DISCUSSION**

Members of the α-crystallin family of molecular chaperones are widespread among prokaryotic and eukaryotic organisms. As stress responsive proteins, they function primarily to prevent irreversible aggregation of proteins during stress conditions. SHsps have been implicated in protective role against a variety of stress such as elevated temperature (Landry *et al.*, 1989; Horwitz, 1992; Plesofsky-Vig and Brambl, 1995; Yeh *et al.*, 1997), low or high pH (Abu Kwaik and Engleberg, 1994; Guzzo *et al.*, 1997; Takeuchi *et al.*, 2003), oxidative stress (Harndahl *et al.*, 1999; Rogalla *et al.*, 1999), metabolic shift from acid to solvent production (Sauer and Durre, 1993). In general, expression of α-crystallin like SHsps is negligible under normal growth conditions but under stress their expression can be increased greatly. The nature of stress which causes this induction might vary from one SHsp to another.
Figure 4.8: Expression levels of sHsp18 in *E. coli* subjected to different stress conditions. sHsp18 levels detected in the immunoblot was quantified using ImageQuant™ as described in materials and methods. The concentration of sHsp18 is given as band volume.

<table>
<thead>
<tr>
<th>Stress stimuli</th>
<th>Aerobic</th>
<th>Microaerobic</th>
<th>Acid stress</th>
<th>H$_2$O$_2$ stress</th>
<th>Ethanol stress</th>
<th>Heat stress</th>
<th>Cold stress</th>
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<tbody>
<tr>
<td></td>
<td>Stationary phase</td>
<td>Mid-log phase</td>
<td>Stationary phase</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>

Table 4.1: Differential expression of sHsp18 across various stress conditions.
that normally survives in a hostile environment of macrophages possesses an α-crystallin like small heat shock protein, sHsp18. Dellagostin et al., (1995) have reported that shsp18 is specifically induced intracellularly in the macrophages. Apart from this study, the response of shsp18 of M. leprae to any other stress condition has not been characterized. Hence, this study was carried out to analyze the shsp18 response to a wide variety of stress conditions, all of which reflect the in vivo stress situations encountered by the pathogen in the host cells.

For this study, shsp18 gene was cloned with the upstream promoter region. The recombinant plasmid was transformed into E. coli and the native promoter driven expression of shsp18 was confirmed. This expressed sHsp18 was found exclusively in the periplasm of E. coli. Recombinant sHsp18 on overexpression has been shown to localize primarily to the outer membrane and to a lesser extent to the periplasm of E. coli (Lini et al., 2008). Subcellular localization of sHsp18 has been reported earlier in M. leprae. Initially, Marques et al., (1998) have shown the presence of sHsp18 in more than one subcellular fraction in armadillo-derived M. leprae, in the membrane as well as the cell wall fraction. Later, this protein was identified in membrane and cytosolic fractions of M. leprae by proteomics approach (Marques et al., 2004). Analysis of sHsp18 sequence does not reveal the presence of any known, characterized protein localization signals. Hence, at this juncture, the mechanism of targeting of sHsp18 to locations other than the cytoplasm is not known.

The objective of cloning shsp18Su in an integrative vector was to integrate it into the genome of a mycobacterial host and to maintain a stable copy of this gene. Analyzing the response of the recombinant mycobacterium harboring a single copy shsp18 to stress would reflect the environment found in M. leprae. However, under the conditions of this study, expression of shsp18 could not be detected in the surrogate host, M. smegmatis. Since it is known that M. smegmatis can utilize the transcriptional start site present 66 bp upstream of the start codon (Dellagostin et al., 1995), the inability to detect sHsp18 in this study may not be because of the lack of recognition of M. leprae promoter. Moreover, earlier studies have also reported a low level expression of shsp18 in mycobacteria (Dellagostin et al., 1995). Hence, the recombinant E. coli carrying shsp18Su, in which expression of shsp18 was confirmed, was taken for stress response analysis. The expression of sHsp18 in E. coli subjected to different stresses, namely microaerobiosis, acid, oxidative, ethanol, heat and cold
stress, was detected, quantified and compared. Induction of \textit{shsp18} was detected under all stress conditions analyzed except acid stress. The induction level increases in the order as:

\textbf{cold stress < heat / ethanol stress < oxidative stress < microaerobiosis < stationary phase.}

\textit{sHsp18} was seen at higher levels during the stationary phase of both aerobic and microaerobic cultures. This implies that \textit{shsp18} is strongly induced as the cells enter the stationary phase. \textit{E. coli} entering the stationary phase, performs an orderly shutdown of metabolism with numerous changes in the cell wall structure (Siegele and Kolter, 1992). One or more of these changes may act as a trigger for \textit{shsp18} induction. This induction of \textit{shsp18} becomes more relevant in the context of \textit{M. leprae} pathogenesis since Young and Cole (1993) have suggested that the normal physiological state of the mycobacterial pathogens during infection resemble more closely to that of \textit{E. coli} entering the stationary phase of growth, when cell survival becomes the priority over cell division.

Microaerobiosis is yet another stress condition in which significant \textit{sHsp18} expression was observed. In cultures grown under microaerobic conditions, \textit{shsp18} induction was prominent even in the log-phase culture and much higher levels of \textit{sHsp18} was observed in the stationary phase cells. It appears that such high levels were achieved in response to the dual signal – low oxygen tension coupled with the transition to stationary phase. These results confirm that low oxygen tension is definitely one environmental cue to which \textit{shsp18} is responsive. Similar induction to hypoxic stress has been reported for the \textit{hspX} of \textit{M. tuberculosis} (Yuan et al., 1996) and \textit{M. bovis BCG} (Cunningham and Spreadbury, 1998).

Microaerobiosis, a low oxygen state, can be correlated \textit{M. leprae} and its life style. Leprosy bacillus is microaerophilic and the concentration of oxygen in the subcutaneous tissue where this pathogen multiplies in the host is very low (Wheeler, 1990). Low oxygen tension has been claimed to be important for growth of \textit{M. leprae} and this organism has been considered to be microaerophilic (Stevens, 1979;Ishaque, 1989;1990). Many efforts were undertaken to grow this pathogen under low oxygen conditions (Chatterjee, 1965;Ishaque, 1989). Franzblau and Harris (1988) showed that when \textit{M. leprae} was maintained under low oxygen concentration, ATP maintenance (an indicator of the overall metabolic status of \textit{M. leprae}) was enhanced.
Moreover, *M. leprae* has been found to persist in granulomas (Cosma et al., 2003), the internal environment of which is also characterized by low oxygen tension (Cunningham and Spreadbury, 1998).

Based on the above observations, it is clear that *M. leprae* seems to be well adapted for survival under microaerobic conditions. This combined with the observation from the present study that microaerobiosis induces *shsp18*, suggests that sHsp18 might play an important role in the adaptation of the leprosy pathogen to hypoxic conditions. Studies by Cunningham and Spreadbury (1998) suggest that the 16 kDa sHsp of *M. tuberculosis* plays a role in stabilizing cell structures during long-term survival which in turn helps the bacilli to survive the low oxygen tension in granulomas. Similarly, sHsp18 may also confer *M. leprae* a survival advantage under microaerobic conditions.

Apart from the microaerobic stress, a modest level of induction was observed in *E. coli* subjected to oxidative stress. This stress reflects the oxidative environment inside the macrophages. One of the bactericidal mechanisms of macrophages is the production of reactive oxidative intermediates such as H$_2$O$_2$. Protection against such ROIs is generally provided by antioxidant enzymes such as superoxide dismutase (SOD), catalase and peroxidase. *M. leprae* is relatively resistant to oxidative killing mechanisms and it has been shown to possess SOD (Thangaraj et al., 1990) and peroxidase activity (Wheeler and Gregory, 1980). Although catalase gene of *M. leprae* is a pseudogene, catalase like activity has also been demonstrated in *M. leprae* (Kang et al., 2001). Wheeler and Gregory (1980) who studied the activities of all the three antioxidant enzymes of *M. leprae* suggested that the enzymological defense against the toxic anions appears incomplete in this pathogen. Additional factors might be employed by *M. leprae* to cope up with the oxidative stress. One such factor is the major glycolipid antigen, PGL-1, which has been shown to scavenge ROI generated in activated macrophages (Neill and Klebanoff, 1988). Although modest, the induction of *shsp18* with H$_2$O$_2$ suggests a probable role for this protein in protection of *M. leprae* against oxidative stress. sHsps have been implicated in protection against oxidative stress (Harndahl et al., 1999; Rogalla et al., 1999) which further strengthens the argument on this protective role of *shsp18*.
The homologs of \textit{M. leprae} shsp18 found in \textit{M. habaana} (now identified as \textit{M. simiae}) and \textit{S. albus} are heat inducible (Lamb \textit{et al.}, 1990; Servant and Mazodier, 1995). In this study, a mild induction of \textit{shsp18} was seen in response to heat shock. In the earlier part of this study, overexpressed sHsp18 was shown to confer thermotolerance to \textit{E. coli} cells when subjected to lethal temperatures. These two results suggest that like many other \textit{shsps}, \textit{shsp18} of \textit{M. leprae} is also inducible by heat.

The ability of \textit{shsp18} to respond to multiple stress stimuli like stationary phase, hypoxia, oxidative stress and heat shock is not unique to this gene. \textit{shsps} of \textit{M. tuberculosis} has also been reported to exhibit this kind of response to multiple stimuli. The tubercle bacilli has two \(\alpha\)-crystallin like \textit{shsps}, \textit{acr1} (hspX) and \textit{acr2}. Expression of \textit{acr1} can be induced by hypoxia (Sherman \textit{et al.}, 2001) or nitric oxide (Voskuil \textit{et al.}, 2003) and is associated with bacterial persistence in a non-replicating state. On the other hand, \textit{acr2} expression is induced by heat shock (Stewart \textit{et al.}, 2002), oxidative stress and during uptake by activated macrophages (Schnappinger \textit{et al.}, 2003). Both these \textit{shsps} display significant stress response and contribute to the persistent infection of \textit{M. tuberculosis} (Stewart \textit{et al.}, 2005). Amongst the mycobacterial species analyzed, \textit{M. leprae} is the only one with a single \textit{shsp} gene. \textit{M. leprae} has undergone massive gene decay (Cole \textit{et al.}, 2001) and has preserved only those genes that are required for its transmission, establishment and survival in the host (Vissa and Brennan, 2001). \textit{shsp18} is one of the preserved functional genes and the retention of \textit{shsp18} suggests that \textit{M. leprae} has a fundamental requirement for this gene. Considering the limited number of functional genes retained by \textit{M. leprae} (Cole \textit{et al.}, 2001), response of \textit{shsp18} to multiple stresses might therefore reflect its functional significance. Induction of \textit{shsp18} was observed as an increase in the levels of sHsp18 protein which in turn implies that the protein may exert a protective effect which may not be directly related to the immediate stress which triggered its synthesis. Instead, it could be possible that sHsp18 by virtue of its molecular chaperone function can act as a general cellular protectant and protect the cells through an enhanced stability of proteins. Therefore, the data presented in this part of the study suggests that \textit{shsp18} have a significant role to play in the stress response of the leprosy pathogen and in turn in its survival and/or persistence under unfavorable conditions.
CONCLUSION

Retention of *shsp18* by *M. leprae* in its genome despite the massive gene decay implies the functional significance of this gene. This was confirmed by the data obtained from the stress response analysis discussed in this part of the study. The results clearly demonstrated that *shsp18* responds to multiple stress stimuli. Hence, it is probable that *M. leprae* might be utilizing the *shsp18* gene along with other factors for its adaptation to different stress conditions encountered at various stages of infection and persistence.