SSUUMMMMAARRYY

Leprosy continues to be a major health problem and the failure to eradicate this disease is closely linked to the pathogenesis of the causative organism, *M. leprae*. Identification and characterization of mycobacterial factors is a prerequisite for understanding the virulence mechanisms of *M. leprae* which in turn contributes to the development of new targets for chemotherapy, immunotherapy and/or prophylaxis. Amongst the mycobacterial genes, those that are selectively expressed during intracellular growth may be involved in intracellular survival and virulence of *M. leprae*. And, *shsp18*, a small heat shock protein gene is one such gene which has been shown to be selectively activated during intracellular growth in the macrophages (Dellagostin et al., 1995) and thus, provided a clue on the prospective role of this gene in the virulence of *M. leprae*. This was further substantiated by the fact that it is the only small heat shock protein gene preserved during the reductive evolution of *M. leprae* (Cole et al., 2001). The present study was designed to gain further insight into the functional aspects of this potential virulence factor. Therefore, the objective of this study was to characterize the *shsp18* gene and gene product particularly with respect to its cellular functions in *M. leprae*.

For this study, the *shsp18* gene was cloned in an *E. coli* overexpression vector and this construct was used to analyze the various characteristics of the recombinant sHsp18. Overexpression levels of sHsp18 in *E. coli* were found to be dependent on the growth temperature as well as the duration of IPTG induction. Maximum levels of sHsp18 were achieved in cells grown at 37 °C at the 4th hour after the addition of IPTG. Further, the observations made from this experiment were used as a basis for determining the various parameters to obtain the desired levels of sHsp18 in subsequent studies. Despite the high levels of protein accumulation on overexpression, sHsp18 does not form inclusion bodies. While a considerable fraction of sHsp18 was retained in the soluble form, the remaining protein was found to be associated with membranes. The overexpressed sHsp18 was found to exist in multiple isoforms. A total of 9 isoforms differing in both molecular mass and pI were observed for the purified protein and the identity of these isoforms were confirmed to be sHsp18 by mass spectrometry.
*M. leprae* survives a diverse range of hostile environments and is exposed to a variety of stress conditions. Stress response is a natural defense mechanism for any organism. Since, sHsp18 in itself is a stress protein, the role of *shsp18* in different stress conditions was analyzed.

Overexpression of any recombinant protein in itself induces a heat-shock like stress condition in *E. coli* and hence, the changes occurring in the host cells in response to sHsp18 overexpression was analyzed. Proteome analysis of *E. coli* overexpressing sHsp18 revealed an upregulation of 34 proteins. Most of these proteins had a role to play in the adaptation of *E. coli* for growth in a complex medium where amino acids are the primary source of carbon as well as to meet the energy demands arising out of high translational activity. In addition, some of the proteins such as ArgS, AmpC were found to be specifically upregulated in response to sHsp18 production. Components of the protein translational machinery have been reported to be downregulated in response to recombinant protein production by other groups. Interestingly, in this study many components of the translational machinery were upregulated. Since rRNA degradation and ribosome destruction are the major factors leading to a decrease in these components, the increase seen here probably indicates that the ribosomal components are prevented from degradation. And, this can be attributed to the protective role of sHsp18, which is a proven molecular chaperone (Lini *et al.*, 2008).

sHsp18 is a heat shock protein and hence, the ability of this protein to protect *E. coli* at elevated temperatures was assessed. Results from the thermal killing assays clearly indicated that sHsp18 was capable of conferring tolerance to *E. coli* when subjected to heat shock. And, a very prominent protective role for this protein was observed at a lethal temperature of 53 °C. At this temperature, *E. coli* cells harboring sHsp18 showed an enhanced survival with a minimum of one log higher number of viable cells when compared to the control. Analysis of the changes occurring in *E. coli* at the protein level during heat shock revealed that some proteins were heat labile and were found to move to the insoluble fraction. The levels of yet another group of proteins were found to be upregulated, which probably represent the heat shock responsive proteins. Further, the level of sHsp18 was found to increase in the insoluble pellet fraction with increase in heat shock and this behavior has been attributed to its inherent molecular chaperone function. It has been proposed that
sHsp18 binds to the heat labile substrates to prevent them from denaturation thereby protecting the *E. coli* during heat shock.

The above studies were carried out under conditions where *shsp18* was expressed under the control of a strong *E. coli* promoter. For subsequent studies, *shsp18* was cloned with an upstream 168 bp promoter region in an integrative vector, pSET152.

Stress response of *shsp18* gene was analyzed using the recombinant *E. coli* harboring this gene with its native promoter by subjecting it to various stress conditions namely microaerobiosis, stationary phase, acid, alcohol, oxidative, heat and cold stress. All stress conditions analyzed reflect the *in vivo* stress encountered by the leprosy pathogen during its infection in the human host. Results from this analysis revealed that *shsp18* responds strongly to the signals during stationary phase and microaerobic growth. A combination of these two conditions was found to result in the highest level of induction of *shsp18*. In addition, oxidative stress and heat shock also results in moderate induction of *shsp18*. The response of *shsp18* to multiple stress stimuli suggests a role for this gene in the adaptation of *M. leprae* to the hostile environments in the host cells.

In order to simulate an *in vivo* situation as in *M. leprae*, the *shsp18* gene with its promoter was integrated in the genome of yet another surrogate host, *M. smegmatis*. Confirmation of this integration event revealed that the pSET152 vector has two different integration sites in this genome. In order to analyze the role of *shsp18* in mycobacterial survival within macrophages, the recombinant *M. smegmatis* carrying *shsp18Su* was taken for infection assays in THP-1 derived macrophage like cells over an infection period of 3-120 hours. Results from these assays indicate that *shsp18* has a role to play in the multiplication of mycobacteria inside the macrophages. Under the conditions of this study, *shsp18* did not seem to alter the phagocytosis of bacteria or prevent the killing by macrophages.

sHsp18 belongs to the α-crystallin family of heat shock proteins which have a monomeric mass in the range of 12-43 kDa (Narberhaus, 2002). However, their functional form is usually an oligomer and recombinant sHsp18 in its native form was also found to exist as an oligomer with 12 monomeric units, a dodecamer. This sHsp18 was shown to exhibit autokinase activity and this ability to autophosphorylate has been demonstrated in very few sHsps. sHsp18 autophosphorylation was both
time and Mg$^{2+}$ ion concentration dependent and the presence of a detergent did not enhance this activity. Intrinsic fluorescence analysis of native, denatured and refolded sHsp18 revealed that these three purified proteins differ in their structural conformation. This difference in the conformation substantiates the presence and absence of autokinase activity in the native and refolded sHsp18, respectively. Further, the fluorescence analysis of sHsp18 before and after autophosphorylation suggests that the addition of phosphate to the protein leads to the dissociation of the oligomers. Demonstration of the kinase activity for sHsp18 opens up exciting possibilities on the ability of M. leprae to modulate the host cell signaling to create a favorable environment for its survival and propagation.

This study has clearly demonstrated the diverse functions of the multifaceted sHsp18 and hence, the significance of shsp18 gene for M. leprae.