MAINTENANCE OF *E. coli* STRAINS

*E. coli* strains were stored for routine use in cold room as single colonies on LB agar plates containing appropriate antibiotics. For long term storage, cultures were preserved in 20% glycerol at -70 °C or as agar stabs at 4 °C.

GROWTH CONDITIONS FOR *E. coli*

All *E. coli* cultures were grown at 37 °C in an orbital shaker or incubator unless mentioned otherwise.

PREPARATION OF *E. coli* COMPETENT CELLS

Competent cells for electroporation were prepared as described by Sambrook *et al.* (1989) with minor modifications. From a freshly streaked plate, a single colony was inoculated in 3 ml of LB broth and grown overnight at 37 °C. From fresh overnight culture, 1% subculturing was done in 100 ml of SOB and grown to an A$_{600}$ of 0.6. The cells were chilled on ice for 30 min and centrifuged at 3000 rpm for 20 min at 4 °C. The cell pellet was resuspended in 100 ml of chilled 10% glycerol prepared in deionized water. The cells were centrifuged at 3000 rpm for 20 min at 4 °C. The pellet was resuspended in 50 ml of 10% glycerol and spun at 3000 rpm for 20 min at 4 °C. After decanting, the pellet was resuspended in the residual glycerol and aliquots (60 µl) were stored in sterile microfuge tubes at -70 °C.

TRANSFORMATION OF *E. coli* CELLS

For electrotransformation, an aliquot of frozen electro-competent cells was thawed on ice. DNA (100 ng) was mixed with the cells and 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; resistance, 400 Ω; capacitance, 25 µF; time, 15 sec that gave a time constant of 5.5 to 7.0. The mixture was diluted in 1ml of SOC and incubated at 37 °C for 45 min for the expression of antibiotic resistance. The culture was then plated on appropriate antibiotic containing LB plates and incubated at 37 °C. The transformants were counted after 12 to 16 h.
OVEREXPRESSION OF sHsp18 IN *E. coli*

Overexpression of sHsp18 was carried out as described by Shabaana (Ph.D Thesis, 2002). M15/pH18 was streaked for single colony in LB agar plate with ampicillin (100 µg/ml) and kanamycin (25 µg/ml). A single colony was inoculated to 3 ml of LB broth containing the appropriate antibiotics and grown overnight at 37 °C at 180 rpm. From fresh overnight culture, 1% subculturing was done to 50 ml of LB medium with antibiotics and grown until A\textsubscript{600} reached 0.6 at 37 °C. At this point, IPTG was added to a final concentration of 0.4 mM and the culture was incubated again at 37 °C for 4 h. After induction, the cells were harvested by centrifugation at 8000 rpm for 30 min at 4 °C and the cell pellet was stored at -20 °C until further use.

**PREPARATION OF THE SOLUBLE AND INSOLUBLE FRACTION OF *E. coli* CELLS**

The *E. coli* cell pellet was resuspended in 0.1 volume of sonication buffer. The cells were lysed by sonication (5 s ON, 5 s OFF for 1 min, 5 cycles at 38% amplitude), and then subjected to low speed centrifugation at 2000 rpm to remove the unbroken cells. The resulting supernatant was then centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant collected represents the soluble fraction (12K supernatant), while the pellet is the insoluble pellet fraction (12K pellet).

**ANALYSIS OF INCLUSION BODY FORMATION**

The presence of overexpressed proteins as inclusion bodies was analyzed based on the method described by Reischl (2003). *E. coli* cells were grown and the protein overexpressed. From 10 ml cells, 12K supernatant and pellet was prepared as described earlier. The 12K pellet was resuspended in 500 µl of sonication buffer containing 2M urea, vortexed for 5 min and incubated in room temperature for 10 min. After centrifugation at 12000 rpm for 7 min, the supernatant was collected. The pellet was sequentially washed as described above with 4 M, 6 M and 8 M urea. The proteins in the supernatant and pellet fractions after each urea extraction step were checked on a 12% SDS-polyacrylamide gel.

**ANALYSIS OF SUBCELLULAR LOCALIZATION OF sHsp18 IN *E. coli***

Subcellular fractionation was performed as described by el Yaagoubi et al. (1994) with the following modifications. *E. coli* culture was grown and 20 ml of the culture
was pelleted. The cell pellet was washed twice in PBS and resuspended in equal volume of spheroplast buffer. After 20 min incubation, cells were spun at 10000 rpm for 15 min. The supernatant (periplasmic fraction) was stored at -20°C. The pellet was resuspended in 2 ml of TE buffer and sonicated. The lysate was centrifuged at 10000 rpm for 4 min at 4°C. The recovered supernatant was again centrifuged at 150000 × g for 40 min at 4°C. The resulting supernatant is the cytoplasmic fraction and was stored at -20°C. The pellet was resuspended in 1 ml of TE buffer with 2% sarkosyl (N-lauryl-sarkosine), incubated at 25°C for 20 min and centrifuged at 50000 rpm for 40 min at 4°C. The supernatant (inner membrane fraction) collected was stored at -20°C while the pellet was resuspended in 200 µl SDS lysis buffer (outer membrane fraction). The proteins in each of the fractions were analyzed by SDS-PAGE.

**MEMBRANE ASSOCIATION OF sHsp18**

Membrane association of the overexpressed protein was checked as per the manufacturer’s instructions (Qiagen Inc.). The overexpressed *E. coli* cells were taken and the 12K pellet prepared as described above. The pellet was resuspended in 500 µl of solubilization buffer containing 0.1 mM EGTA and 0.25% Tween-20 and vortexed vigorously for 5 min. After incubating at room temperature for 10 minutes, it was centrifuged at 10000 rpm for 5 min. The supernatant and pellet fractions were collected and checked by SDS-PAGE.

**HEAT SHOCK EXPERIMENTS WITH *E. coli***

Thermotolerance experiments were carried out based on the method described by Muchowski and Clark (1998). A single colony of *E. coli* was inoculated to 3 ml LB broth with ampicillin and kanamycin and incubated at 37°C for overnight. To 50 ml of fresh medium with antibiotics, 1 % sub culturing was done using the fresh overnight culture and incubated at 37°C till the culture reached A600 of 0.6. IPTG was added at 0.4 mM and the culture was incubated at 37°C for 2 h. After taking an aliquot of the cells (0 time point of heat shock), the culture was shifted to one of the heat shock temperature (46°C, 50°C or 53°C). Every 30 min, an aliquot of the culture was taken for 120 min. For survival assays, at every time point the culture was serially diluted ten-fold in 0.9% saline and 100 µl of the selected dilution was
plated on LB agar. The plates were incubated at 37 °C and the number of colony forming units was determined after 12 to 16 h. For protein studies, the cells were pelleted and the soluble and insoluble protein fraction prepared as described above.

ANALYSIS OF shsp18 INDUCTION UNDER STRESS STIMULI

A single colony of XL1/pSET-18Su from a freshly streaked plate was inoculated to 3 ml of LB with apramycin (50 µg/ml) and incubated at 37 °C, overnight. To 50 ml (6 sets) of LB with apramycin, 2 % subculturing was done and the culture was grown till the A600 reached 0.5. Cultures were treated as follows for analysis of stress response (Yuan et al., 1996; Cunningham and Spreadbury, 1998)

1. **Stationary phase**: The culture was grown at 37 °C to A600 of 1.6
2. **Acid stress**: The culture was pelleted at 2500 rpm at 4 °C for 15 min. The cells were resuspended in 50 ml of LB broth at pH 4.0 and incubated at 37 °C
3. **Oxidative stress**: Hydrogen peroxide was added to the culture to a final concentration of 10 mM and incubated at 37 °C
4. **Alcohol stress**: Ethanol was added at 5% and incubated at 37 °C
5. **Heat stress**: The culture was shifted to 50 °C
6. **Cold stress**: The culture was incubated in ice

The cultures were maintained in the respective stress condition (except stationary phase culture) for 30 min.

7. **Microaerobic growth**

Microaerobic conditions were simulated by growing cultures in conical flask with 4/5th volume filled with the medium as against the 1/5th medium volume used for normal aerobic cultures (Narro et al., 1990). For microaerobic cultures, from the same primary culture, 2% subculturing was done to 200 ml of LB in a 250 ml conical flask and incubated at 37 °C at 180 rpm. An aliquot of the culture was removed when the OD at 600 nm reached 0.5 (Mid-log phase culture under microaerobic conditions). The culture was further incubated at 37 °C until it reached an OD of 1.6 which represents the stationary phase culture grown under microaerobic conditions.
At the end of the stress stimuli, the cells were harvested and resuspended at 1 OD/100 µl in SDS-lysis buffer. The cells were lysed by boiling at 100 °C for 3 min.

**MAINTENANCE OF *M. smegmatis***

*M. smegmatis* cultures were stored for routine use in cold room as single colonies on LB or BHI agar plates containing appropriate antibiotics. For long term storage, cultures were preserved in 20% glycerol at -70 °C.

**GROWTH CONDITIONS FOR *M. smegmatis***

*M. smegmatis* cultures was grown in LB or Brain heart infusion medium at 37 °C in an orbital shaker or incubator. Glycerol (0.2%) and Tween-80 (0.1%) were added to the medium.

**PREPARATION OF *M. smegmatis* COMPETENT CELLS**

Competent cells for electroporation were prepared as described by Snapper *et al.* (1990) with minor modifications. Fresh overnight cultures were made in BHI broth and then subcultured in 100 ml of BHI broth. The culture was grown to an A_{600} of 0.3. The cells were chilled on ice for 2 h and centrifuged at 3000 rpm for 20 min at 4 °C. The pellet was resuspended in 100 ml of 10% glycerol, prepared using deionized water. The cells were centrifuged at 3000 rpm for 20 min at 4 °C. The pellet was resuspended in 50 ml of 10% glycerol and spun at 3000 rpm for 20 min at 4 °C. After decanting, the pellet was resuspended in the residual glycerol and aliquots (60 µl) were stored in sterile microfuge tubes at -70 °C.

**TRANSFORMATION OF *M. smegmatis* CELLS**

For electrottransformation, an aliquot of frozen electro-competent cells was thawed on ice. One microgram of DNA was mixed with the cells and 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; resistance, 1000 Ω; capacitance, 25 μF; time, 15 sec that gave a time constant of 8.0 to 10.0. The electroporated cells were immediately diluted in 2 ml of BHI broth and kept on a shaker at 37 °C for 3 h for the expression of antibiotic resistance. The culture was then plated on appropriate antibiotic containing BHI plates and incubated at 37 °C. The transformants were counted after 3 to 5 days.
ACID FAST STAINING

Acid fast staining was done as described by Smithwick (1976). A smear of the mycobacterial culture was made on a clean glass slide, air dried and heat fixed. Carbol fuchsin stain was added on the smear and the slide was heated gently under the flame three times over a period of 5 min. The decolorizing solvent (25% sulfuric acid) was then gently added over the smear and incubated for 1 min. The slide was washed with water and counterstained by adding 1% methylene blue stain on the smear and left for 2 min. The slide was washed well with running tap water and examined under 100X oil immersion lens.

ISOLATION OF PLASMID DNA

Plasmid DNA was isolated by alkaline lysis method (Sambrook et al., 1989) with minor modifications. Log phase cells grown in 3 ml of LB containing appropriate antibiotics were centrifuged and resuspended in 100 μl TEG buffer and incubated at room temperature for 5 min. To this, 200 μl of alkaline solution (0.2 N NaOH, 1% SDS) was added, the contents were gently mixed and incubated in ice for 5 min. To this, 150 μl of 3 M potassium acetate (pH 4.8) was added, mixed gently and left on ice for 10 min. RNase (100 μg/ml) was added and incubated at 37 °C for 20 min. After centrifuging at 12000 rpm for 10 min, equal volumes of chloroform:isoamyl alcohol (24:1) was added to the supernatant. Mixed well, centrifuged at 12000 rpm for 2 min and the supernatant was transferred to fresh microfuge tube. Equal volumes of isopropanol was added and left at -20 °C for 30 min. The DNA was pelleted by centrifugation at 12000 rpm for 10 min, washed once with 70% ethanol, dried and dissolved in 50 μl of TE.

ISOLATION OF TOTAL DNA FROM M. smegmatis

Genomic DNA was isolated from M. smegmatis by method described by Husson et al. (1990) with modifications. One gram wet weight of M. smegmatis cells were taken and resuspended in 5 ml of sucTE. Lysozyme was added at 3 mg/ml final concentration and the cells were incubated at 37 °C for 1.5 h. The cell suspension was trituriated every 15 min. To this, 1.2 ml of 0.5 M EDTA and SDS to 1% was added and mixed carefully. Pronase was added at 150 μg/ml and incubated at 37 °C for 40 h. Six millilitre of TEN-phenol was added and mixed carefully for 10 min. To
this, 6 ml of chloroform was added, mixed well for 5 min and centrifuged for 20 min at 3000 rpm. The aqueous phase was collected and 3 ml of TEN buffer was added to the phenol phase. After mixing, it was centrifuged at 3000 rpm for 20 min and the aqueous phase was collected and pooled with the earlier one. To the aqueous phase, equal volumes of chloroform:isoamylalcohol (24:1) mixture was added, mixed well and centrifuged at 3000 rpm for 20 min. Extraction with chloroform was repeated once more. To the aqueous phase, 0.1 volume sodium acetate and 2.5 volumes of ethanol was added to precipitate out the DNA. The DNA was recovered and washed once with 70% ethanol and air dried briefly. DNA was dissolved in 1 ml of TE and stored at 4 °C.

**ISOLATION OF M. leprae GENOMIC DNA**

*M. leprae* infected armadillo tissue were fixed in formaldehyde and embedded in wax (Shabaana *et al.*, 2001). Thirty 10 micron sections were collected in a microfuge tube and deparaffinized using xylene. The pellet was washed with 70% ethanol and dried under vacuum. After adding 1 ml of lysis buffer, lysozyme and proteinase K were added to a final concentration of 2 mg/ml and 300 µg/ml, respectively. Samples were sonicated in a sonicator bath at 80% amplitude for 5 min. Samples were incubated for 6 h at 37 °C and treated with 1 µl of RNase (10 µg/µl) for 20 min at 37 °C. Samples were boiled for 10 min and DNA was extracted using phenol:chloroform (1:1), and precipitated using isopropanol. DNA concentration and purity was determined by measuring the absorbance at 260/280 nm using spectrophotometer.

**EXTRACTION OF RNA**

Biopsy materials were fixed in formaldehyde and embedded in wax (Shabaana *et al.*, 2001). Thirty, 10 µm sections were collected and were deparaffinized in xylene and samples were incubated in digestion buffer for 24 h at 52 °C followed by Trizol extraction. RNA was treated with DNase according to the manufacturer’s instructions (Promega). The concentration of RNA was checked using a spectrophotometer.

**PREPARATION OF cDNA**

Total RNA (3 µg) was reverse transcribed in a final volume of 50 µl using a conventional programmable thermal cycler. The reaction mix containing RNA, 1 µg oligo dT15 and 1 µg random hexamers (Promega) was incubated at 70 °C for 10 min and chilled on ice. Master mix containing 10 µl M-MLV reaction buffer (5X ), 2.5 µl
dNTP mix (10 mM each), 2 µl RNase inhibitor (20 U/µl) and 200 U M-MLV reverse transcriptase for 1 h. The enzyme was heat-inactivated at 92 °C for 2 min and cDNA was stored at -20 °C.

**POLYMERASE CHAIN REACTION**

PCR amplification of the template DNA was carried out using thermal cycler PTC 200 (MJ Research). Cycling parameters were: initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min for 36 cycles followed by a final extension for 7 min. PCR was performed in a 25 µl reaction mix containing 5 µl of cDNA, 2.5 µl 10X buffer (final concentration of 1X), 1.25 units of Taq Polymerase, 2 µl of MgCl₂ (final concentration of 1 mM) and 2 µl of dNTPs (final concentration of 100 µM). PCR products were electrophoresed on agarose gel and visualized by ethidium bromide staining.

**AGAROSE GEL ELECTROPHORESIS**

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook *et al.* (1989). Required amount of agarose (depending on the percentage) was melted in distilled water taken in a beaker. The molten agarose was kept in 50 °C water bath for 10 min and 50X TAE was added to give a final concentration of 1X TAE. The agarose solution was then poured on to a gel template sealed with tape and fitted with comb. The agarose was allowed to set to gel for 30 min. The comb was then removed and the gel was immersed in 1X TAE buffer in horizontal electrophoresis tank. DNA samples were mixed with 1/6 volume of 6X loading buffer and electrophoresed at 5 V/cm. Lambda DNA digested with *Hind*III or 1 kb DNA ladder was run in parallel as a size standard.

**Staining the gel**

After the electrophoresis was over, the gel was stained using ethidium bromide solution (0.5 µg/ml) for 30 min. The gel was then briefly destained in water. The bands were visualized using short wave (300 nm) transilluminator and photographed with a gel documentation system.

**RESTRICTION AND LIGATION**

Restriction endonuclease digestion and ligation were carried out according to the manufacturer’s instructions.
PLASMID RESCUE STRATEGY

The integration of \textit{shsp18Su} gene in the genome of \textit{M. smegmatis} was confirmed by a plasmid rescue strategy as described by Paranthaman (Ph.D Thesis., 2003). The presence of a single \textit{Bam}HI site in pSET152 plasmid DNA allowed the cloning of one of the phage/host hybrid integration sites as described below. One microgram of genomic DNA from a transformant was digested with \textit{Bam}HI. The digested DNA was purified and self ligated with T4 DNA ligase. Ligated total DNA was used for transforming \textit{E. coli}, XL1 Blue MRF’. Apramycin resistant transformants were selected and the plasmid DNA was isolated. Restriction analysis was carried out to check for the presence of vector as well as chromosomal DNA.

TOTAL PROTEIN ASSAY

Protein concentration was determined by Bradford’s estimation or Lowry’s estimation method using bovine serum albumin as the standard.

**Bradford estimation** (Bradford, 1976)

The protein sample was diluted to 1 ml with deionized water. Five milliliter of Bradford working solution was added, mixed by vortexing and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm, setting blank against deionized water.

**Lowry estimation** (Lowry et al., 1951)

The protein sample was diluted to 1 ml with deionized water. Four milliliter of alkaline copper reagent was added, vortexed and incubated at room temperature for 10 min. To this, 0.5 ml of Folin-ciocateau reagent was added, vortexed and incubated at room temperature in dark for 20 min. The absorbance was measured at 750 nm, setting blank against deionized water.

PURIFICATION OF HIS-TAG PROTEIN

**Denaturing purification**

Induced \textit{E. coli} culture pellet stored at -20 °C was thawed in ice for 30 min. The cells were resuspended in 0.1 volume of buffer B, mixed well and incubated at 30 °C with shaking for 60 min for the cell lysis to occur. The cells were then centrifuged at
8000 rpm for 15 min at 4 °C and the supernatant was collected. The supernatant was loaded on the column packed with 1 ml of Ni-NTA slurry equilibrated with buffer B. The cell lysate was allowed to flow through the column under gravity. The column was washed with 10 column volumes of buffer B and subsequently with 6 column volumes of buffer C, thrice. The bound proteins were finally eluted with 5 volumes of elution buffer and the eluted protein was stored at 4 °C.

**Refolding of denatured protein**

The protein purified under denaturing conditions using urea was dialyzed against the refolding buffer (Arora and Khanna, 1996). The dialysis was carried out for 16 h at 4 °C. The refolded protein was stored as aliquots at -70 °C.

**Non-denaturing purification**

sHsp18 was purified under non-denaturing conditions as per manufacturer’s instructions (Qiagen Inc.) with slight modifications. The *E. coli* cells were grown at 30 °C and sHsp18 expression was induced for 4 h as described above. At the end of 4 h, the cells were chilled on ice for 30 min and then harvested by centrifugation at 8000 rpm at 4 °C for 30 min. After the cells were washed twice with PBS and after determining the wet weight of the cells, they were frozen at -20 °C.

The frozen cell pellet was thawed in ice for 30 min and resuspended in sonication buffer at 5 volumes per gram of wet weight. To this, 1 mg/ml of lysozyme was added and incubated in ice for 30 min. The cells were sonicated at 35% amplitude for one minute pulse (5 s ON and 5 s OFF) for a total of 5 cycles. The cell lysate was drawn through a 21 gauge needle attached to a 20 ml syringe 2-3 times and then centrifuged at 10000 rpm for 20 min at 4 °C. The supernatant was mixed with 1 ml of Ni-NTA slurry equilibrated with sonication buffer and incubated in ice on a belly shaker to allow the binding of His-tag protein to the matrix. The batch binding was carried out for 60 min after which the cell lysate along with the matrix was loaded on to the column carefully avoiding trapping of air bubbles. The soluble fraction of the cell lysate was allowed to flow through the column under gravity as the matrix got packed in the column. After the flow through, the column was washed with 40 ml of sonication buffer followed by wash with 40 ml of buffer containing 20 mM imidazole at a flow rate of 0.6 ml/min. After the washes, the proteins were eluted using elution buffer with increasing concentrations of imidazole (100 mM, 150 mM, 200 mM,
250 mM and 500 mM). For every concentration of imidazole, 5 ml of the elution buffer was passed through the column at 3 column volume per hour and 1 ml fractions were collected. The fractions were stored at -20 °C. The purification profile was followed by loading equal volumes of all the fractions collected during the purification steps on a 12% SDS-PAGE.

RAISING ANTI-sHsp18 ANTIBODIES IN RABBIT

Antibody against purified sHsp18 was raised in rabbit as described by Coligan et al. (1992). Five hundred microliter of purified protein containing 300 µg of protein was mixed with 500 µl Freunds’s complete adjuvant (Sigma). The adjuvant was rapidly mixed in syringe and the resulting uniform suspension was injected at four different places in the hind portion of a rabbit. Blood (2 ml) was taken from the animal’s ear vein before immunizing to serve as normal rabbit serum (pre-immune serum). On the 28th day after the primary immunization, a booster dose was given. Five hundred microliter of protein sample containing 500 µg of protein was rapidly mixed with 500 µl of Freund’s incomplete adjuvant in syringe and injected immediately as described for primary immunization. Blood was collected from animal’s ear vein on 5th day after the booster dose.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were resolved in SDS-polyacrylamide gel (Laemmli, 1970) as described by Ausubel et al. (1989). Routinely, 0.75 mm thick gels were cast in a protein gel apparatus (Hoefer or Yercaud Biotech) using a separating gel (12%) and a stacking gel (4.5%). The separating gel solution was first poured into a sealed glass plate cassette. Water saturated n-butanol was layered gently on top of the separating gel and the gel was set to polymerize for 30 min at room temperature. After polymerization the butanol layer was removed carefully and the exposed end was rinsed with 0.1% SDS. Stacking gel solution was poured onto the separating gel up to the cassette leaving 1 cm. Comb was inserted into the cassette leaving 1 cm distance between the bottom of the slots and top of the separating gel. After the polymerization of the stacking gel, the comb was carefully removed. The slots were cleaned by rinsing with electrophoresis buffer. Protein samples were loaded into the slots and the slots were filled with 1X electrophoresis buffer. A constant current of 15 mA/gel was applied to electrophorese the samples.
**BIS-TRIS POLYACRYLAMIDE GEL**

Proteins in native state was resolved in Bis-tris polyacrylamide gel (Engelhorn, 1996) without any reducing agent. Routinely, 0.75 mm thick gels were cast using a separating gel (7%) and a stacking gel (4%). The separating gel solution was first poured into a sealed glass plate cassette. Water saturated n-butanol was layered gently on top of the separating gel and the gel was set to polymerize for 2 h at room temperature. After polymerization, the stacking gel solution was poured onto the separating gel up to the cassette leaving 1 cm. Comb was inserted into the cassette leaving 1 cm distance between the bottom of the slots and top of the separating gel. Alternatively, a single separating gel of 6.5% was cast without any stacking and the comb was placed. The gel was allowed to polymerize for 2 h. After the polymerization, the comb was carefully removed. The slots were cleaned by rinsing with electrophoresis buffer. Protein samples were loaded into the slots and the slots were filled with 1X electrophoresis buffer and a constant current of 15 mA/gel was applied.

**COLLOIDAL COOMASSIE STAINING**

The gels were stained as described by Candiano *et al.* (2004). After the completion of electrophoresis, the gel cassette was dismantled and the gel was immersed in fixing solution for 1 h. The fixing solution was removed and the gel was washed in deionized water for 10 min for three times. The gel was then placed in colloidal coomassie blue staining solution and was agitated slowly for overnight. The gel was destained by repeated water washes until there was no background and the gel was documented.

**WESTERN BLOTTING**

Protein samples separated in 12% SDS-PAGE were blotted onto nitrocellulose membranes (Sigma) using Hoefer semidry electroblotting apparatus as described by Ausubel *et al.* (1989). After electrophoresis, the gel was soaked in chilled Towbin buffer for 20 min. The membrane was soaked in Towbin transfer buffer was placed over three sheets of Whatmann 3 mm filter papers kept on bottom plate (anode). Proper care was taken to avoid trapping air bubbles between the layers of filter papers. Gel was placed on top of the membrane, followed by three sheets of pre-wet
filter papers and air bubbles were removed by rolling a pipette on top of the filter papers. The top plate (cathode) and safety cover were placed and blotting was carried out at 0.8 mA/cm² for 2 h. After blotting, the membrane was carefully removed and rinsed in TBS and used immediately.

**Immunodetection**

After transfer, the membrane was rinsed in TBS and blocked with 5% milkpowder in TBS-T for 2 h. The blocking solution was replaced with anti-sHsp18 antibody (1:1000 dilution) in TBS containing 0.1% milkpowder (first antibody solution) and kept on a shaker for overnight at 4 °C. The first antibody solution was poured off and the membrane was washed thrice with TBS-T followed by three washes with TBS. Goat anti-rabbit antibody (1:1000 dilution) in TBS containing 0.1% milkpowder was added and kept on shaker for 45 min. After washing thrice with TBS-T, the substrate solution was added and kept in dark until the bands appeared. After sufficient color development, the substrate solution was poured off and the membrane was rinsed with distilled water several times and documented.

**TWO DIMENSIONAL GEL ELECTROPHORESIS**

**E. coli** total cell lysate preparation for 2D PAGE

*E. coli* cells were grown and sHsp18 overexpressed as described earlier. The cells were pelleted and washed twice with cell wash buffer. The cells were then resuspended in cell lysis buffer at 1 OD cells/100 µl and subjected to sonication (5 s ON, 10 s OFF; 10 pulses at 25% amplitude). The cell suspension was centrifuged at 12000 rpm for 10 min at 4 °C and the supernatant was stored at -70 °C until further use for 2D experiments.

**2D PAGE**

Samples were diluted in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes of the pH range same as that of the IPG strips, 50 mM DTT and 0.004% bromophenol blue. Samples were then applied on to a 7 cm or 18 cm, pH 4-7 linear IPG strip and focused at a temperature of 20 °C using the IPGphor IEF apparatus as per the following protocol:
For 7 cm IPG strips

Step 1: 300 V, Step and Hold for 1 h
Step 2: 1000 V, Gradient for 1 h
Step 3: 5000 V, Gradient for 1 h
Step 4: 5000 V, Step and Hold for 4 h

For 13 cm IPG strips

Step 1: 500 V, Step and Hold for 1 h
Step 2: 1000 V, Gradient for 1 h
Step 3: 5000 V, Gradient for 2 h
Step 4: 5000 V, Step and Hold for 6 h

For 18 cm IPG strips

Step 1: 0 V, Rehydration for 1 h
Step 2: 30 V, Step and Hold for 11 h
Step 3: 1000 V, Gradient for 1 h
Step 4: 5000 V, Gradient for 2 h
Step 5: 8000 V, Gradient for 1 h
Step 6: 8000 V, Step and Hold for 7 h

The focused strips were stored at -70 °C until the second dimension analysis or used immediately. The frozen strips were brought to room temperature and subjected to two-step equilibration. The proteins were initially reduced with 2% DTT in equilibration buffer for 15 min followed by alkylation with 2.5% IAA in equilibration buffer for 15 min. Strips were then layered on top of a 12.5% polyacrylamide gel and sealed with 0.5% agarose in electrophoresis buffer. DoDeca apparatus (Bio-Rad) (for 7 cm strips) or Sturdier system (Hoefer) (for 13 cm strips) or DALT six apparatus (GE Healthcare) (for 18 cm strips) was used for second dimension electrophoresis and the proteins were separated using the following power settings

For 7 cm: 100 V, 400 mA, 10 W for 1.5 h

For 13 cm: 10 mA (constant current)
For 18 cm: 20 W for 30 min and then 60 W for 5 h

After completion of electrophoresis, the gels were stained with colloidal coomassie stain.

**SAMPLE PREPARATION FOR MALDI-TOF MASS SPECTROMETRY**

**In-solution digestion**

Hundred picomoles of the purified protein were taken and the volume was made up to 10 µl using ammonium bicarbonate in 10% acetonitrile. The final concentration of ammonium bicarbonate should be 50 mM. If the protein solution was in buffer containing urea, it was incubated at 37 °C for 10 min. In other cases, the protein was kept in boiling water bath for 5 min and slowly cooled to room temperature. Trypsin was added at 400 ng concentration in 5 µl. The sample was incubated at 37 °C for 16 h. The peptides were desalted by zip tip purification.

**In-gel digestion**

The identification of proteins by MALDI-TOF mass spectrometry was done as described in Gupta *et al.* (2007).

**Destaining of 1D protein bands**

The selected protein bands were excised out and 0.5 cm of this band was taken for processing. The protein band was cut to 1 mm² small pieces. The colloidal coomassie stained gel pieces were first washed two times in water for 10 min each. The gel pieces were destained by incubation in 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) for 15 min. This step was repeated until the gel pieces were completely destained. Subsequently, the gel pieces were dehydrated in 100% acetonitrile for 15 min and dried under vacuum for 30 min. After rehydrating the gel pieces in 50 µl of 10 mM DTT in 25 mM ammonium bicarbonate, it was incubated at 56 °C water bath for 30 min. It was incubated at room temperature and 50 µl of 55 mM IAA in 25 mM ammonium bicarbonate was added. The gel pieces were incubated at room temperature in dark for 30 min. After removing the solution, 100 µl of 100% ACN was added to the gel pieces and incubated for 15 min. Subsequently, the gel pieces were washed thrice with 100 mM ammonium bicarbonate.
bicarbonate, 20 min each. The gel pieces were dehydrated with 50 µl of ACN and dried under vacuum for 30 min. The dried gel pieces were taken for tryptic digestion.

**Destaining of 2D protein spots**

Selected protein spots were excised manually from the 2D gel using a metal punch. This gel piece was further cut into smaller 1 mm² pieces. The colloidal coomassie stained gel pieces were first washed two times in water for 10 min each. The gel pieces were destained by incubation in 25 mM ammonium bicarbonate made in 50% acetonitrile for 15 min. This step was repeated until the gel pieces were completely destained. Subsequently, the gel pieces were dehydrated in 100% acetonitrile for 15 min and then dried under vacuum for 30 min. The dried gel pieces were taken for tryptic digestion.

**In-gel tryptic digestion**

Dried gel pieces were rehydrated for 30 min on ice with 5 µl of trypsin (80 ng/µl) in 100 mM ammonium bicarbonate in 10% acetonitrile. The gel pieces were overlaid with 20 µl of 40 mM ammonium bicarbonate in 10% acetonitrile and incubated at 37 °C water bath for 16 h.

**Extraction of peptides**

The tubes containing the digested peptides were then briefly centrifuged for 5 s and the supernatant containing the peptides was saved. Peptides were extracted from gel pieces using 25 µl of 0.1% trifluoroacetic acid (TFA) made in 60% acetonitrile by sonication for 3 min at a frequency of 2200 MH in Soltech Ultrasonic cleaner. The tubes were then centrifuged for 5 s and incubated for 10 min. Supernatant obtained after centrifugation was pooled with the first supernatant. Twenty microliters of 100% acetonitrile was added to gel pieces for dehydration and vortexed. The tubes were then centrifuged for 5 s and incubated for 10 min. This supernatant was combined with the earlier two supernatants and these extracted peptides were vacuum dried for 60-90 min. Dried peptides were taken for zip-tip purification.

**Peptide purification**

The samples were concentrated and desalted using eppendorf Perfect pure C18 tips. The tip was loaded onto a pipette set to ten µl and the plunger was depressed to a dead
stop (this was followed in between all steps too). Dried peptides were resuspended in 5 µl of 0.1% TFA in 5% acetonitrile. The C18, 10 µl tip was first wetted by aspirating the wetting solution in and out for 7 times. The column was then equilibrated by aspirating the equilibration solution, 7 times. The resuspended peptides were then allowed to pass through the column by aspirating it in and out for 10 times. The column was subsequently desalted by allowing the equilibration solution to pass through 3-4 times. Finally, the peptides were eluted in 5 µl of elution solution (50% ACN in 0.1% TFA).

MALDI-TOF MASS SPECTROMETRY OF TRYPTIC PEPTIDES

Matrix preparation

One ml of matrix resuspension solution was prepared as follows. To a 10 mg vial of lyophilized matrix, 600 µl of the resuspension solution was added and vortexed thoroughly. The vial contents were transferred to a 1.5 ml microfuge tube. Remaining 400 µl was added to the vial to dissolve the remaining matrix. The vial was vortexed again and the contents were added to the earlier 600 µl in the microfuge tube. The matrix should have straw yellow color. The matrix solution was briefly centrifuged at 10000 rpm for 10 s, to allow the undissolved matrix crystals to settle down. The clear supernatant was distributed in 0.5 ml tubes in aliquots of 100 µl and stored in dark at 4 °C.

Preparation of peptide mix standards

Each sigma vial has 10 nmol of a standard peptide in lyophilized powder form. The peptides were reconstituted into a main stock (100 pm/µl) by adding 100 µl of the respective solvents as given in the table below and stored at -20 °C. Working solution (10 pm/µl) was made by mixing 10 µl of the main stock with 90 µl of the respective solvent and stored at -20 °C.
The peptide mix was prepared in the following way:

<table>
<thead>
<tr>
<th>Standard Peptide</th>
<th>Mono isotopic mass in Daltons</th>
<th>Solvent</th>
<th>Final conc (pm/µl)</th>
<th>Final conc (pm in 20 µl)</th>
<th>Volume for 20 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradykinin (Fragment 1-7)</td>
<td>757.3997</td>
<td>50% ACN in 0.05% TFA</td>
<td>4.5</td>
<td>90</td>
<td>9 µl of 10 pm/µl</td>
</tr>
<tr>
<td>Angiotensin II (Human)</td>
<td>1046.5423</td>
<td>0.1% TFA</td>
<td>2.0</td>
<td>40</td>
<td>4 µl of 10 pm/µl</td>
</tr>
<tr>
<td>P14R (synthetic peptide)</td>
<td>1533.8582</td>
<td>0.1% TFA</td>
<td>1.5</td>
<td>30</td>
<td>9 µl of 10 pm/µl</td>
</tr>
<tr>
<td>ACTH (fragment 18-39)</td>
<td>2465.1989</td>
<td>0.1% TFA</td>
<td>1.5</td>
<td>30</td>
<td>9 µl of 10 pm/µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>19 µl</strong></td>
</tr>
</tbody>
</table>

For making the final peptide mix, to the above 19 µl, one µl of 100% ACN:0.1% TFA (30:70 ratio) was added.

**Preparation of protein standards**

Each sigma vial has 10 nmol of a standard protein in lyophilized powder form. The peptides were reconstituted into a main stock (100 pm/µl) by adding 100 µl of the respective solvents as given in the table below and stored at -20 °C. Working solution (10 pm/µl) was made by mixing 10 µl of the main stock with 90 µl of the respective solvent and stored at -20 °C.

<table>
<thead>
<tr>
<th>Standard Peptide</th>
<th>Average mass in Daltons</th>
<th>Solvent</th>
<th>Final conc (pm/µl)</th>
<th>Final conc (pm in 30 µl)</th>
<th>Volume for 30 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>12361.96</td>
<td>0.1% TFA</td>
<td>3.3</td>
<td>100</td>
<td>10 µl</td>
</tr>
<tr>
<td>Aldolase</td>
<td>16952.27</td>
<td>0.1% TFA</td>
<td>3.3</td>
<td>100</td>
<td>10 µl</td>
</tr>
<tr>
<td>Apomyoglobin</td>
<td>39212.28</td>
<td>0.1% TFA</td>
<td>3.3</td>
<td>100</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Spotting methods

Sandwich method (Dai et al., 1999)

An initial layer of matrix (0.5 µl) was spotted on the target plate and allowed to dry. Sample (0.5 µl) was applied and then 0.5 µl of matrix was layered immediately on top so that the peptides co-crystallize with the matrix. This method of spotting was used for CHCA matrix.

Dried droplet (Karas and Hillenkamp, 1988)

One volume of sample was mixed with 2 volumes of matrix and 1 µl was spotted on the target plate. This method of spotting was used for DHBA matrix.

Amp method (Zhu and Papayannopoulos, 2003; Smirnov et al., 2004)

Sample was diluted 5-fold (0.5 µl of sample with 4.5 µl of matrix) in CHCA matrix containing ammonium phosphate. One µl of this mix was then spotted on the target plate.

Calibration of mass spectrometer

For calibrating the instrument, the peptide mix containing four peptides of known molecular mass was spotted in the middle of four samples. The peptide mix was spotted the same way as the sample depending on the matrix used. All the samples were spotted in duplicates for every matrix used. After spotting, the samples were dried in a dryer for a minimum of 20 minutes.

MALDI spectrum acquisition

Peptide Mass Fingerprint (PMF)

The MALDI target plate was loaded on to the MALDI-TOF instrument and the vacuum was allowed to build. Peptide mass spectrum was acquired in the reflectron mode. The acceleration voltage after pulsed extraction was 20000. The instrument was calibrated using external standards, Bradykinin (757.39 Da), Angiotensin II (1046.54 Da), P14R (1533.85 Da) and ACTH fragment 18-39 (2465.19 Da). The monoisotopic peak list was generated with Kratos LAUNCHPAD™ software.
version 2.4, without using the smoothing function and the peak filter was applied to exclude masses lower than 750 Da.

**DATABASE SEARCH FOR PROTEIN IDENTIFICATION**

The peaks with a signal-to-noise ratio of 20 and above were used for database search. The monoisotopic masses were taken for identification by using the search engine, MASCOT (www.matrixscience.com). Search was performed in NCBInr, MSDB and Swissprot databases. The search parameters were as follows: tolerance – 0.05 Da to 0.5 Da; Species – *M. leprae* or *E. coli* as applicable; maximum number of missed cleavages – 1; fixed modification – carbamidomethyl (for cysteine modification by iodoacetamide); variable modifications – oxidation of methionine, propianomide (for cysteine modification by acrylamide), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E). A protein would be considered as identified if the MOWSE score exceeds the threshold score mentioned for each search (*p* < 0.05).

**PROTEIN MOLECULAR MASS ANALYSIS**

The purified protein was mixed with equal volumes of sinnapinic acid matrix and 1 µl of this mixture was spotted on the MALDI target plate. The whole protein calibration mix was also spotted similar to that of the sample and was dried in an air dryer for 20 min. The MALDI target plate was loaded on to the MALDI-TOF instrument and vacuum was allowed to build. Whole protein mass spectrum was acquired in the linear mode. The acceleration voltage after pulsed extraction was 16000. The instrument was calibrated using external standards, cytochrome C (12361.96 Da), apomyoglobin (16952.27 Da) and aldolase (39212.28 Da). The average molecular mass of the protein sample was generated with Kratos LAUNCHPAD software version 2.4, using the smoothing function.

**LC-MS/MS ANALYSIS OF TRYPpic PEPTIDES**

The dried peptides were resuspended in 0.1% formic acid and taken for analysis. A nanoflow ESI source was used with a lockspray source for lockmass measurement during all the chromatographic analysis. The peptides were separated in Acclain PepMap 100 C18 capillary columns by elution with 80% acetonitrile in water and 0.1% formic acid gradient. Data were acquired in a data dependent mode and multiply charged peptide ions were automatically mass selected and dissociated in
MS/MS experiments. Typical LC and ESI conditions used for analysis were: a flow rate of 220 nl/ml, nanoflow capillary voltage of 1500 V, block temperature of 100 °C and cone voltage of 170 V. MS spectra were processed using Data Analysis v3.4 software and the generated mgf files were used to perform the database search using the MASCOT search engine.

ANIMAL CELL CULTURE TECHNIQUES

THP-1 cells

Reviving

A single vial of THP-1 frozen stock was removed carefully from the liquid nitrogen storage can following proper precautions. The vial was swirled gently for a minute in a beaker containing water prewarmed to 37 °C. When some part of the cells was still frozen, the cells were transferred to 50 ml RPMI medium to dilute the DMSO. Cells were then spun at 700 rpm for 5 min and the supernatant was discarded. The cells were resuspended very gently with RPMI medium and were transferred to multiple wells in a 6-well plate (with 3 ml medium) and/or a 25 cm² culture flask (with 5 ml of medium). The cells were incubated in a humidified chamber with 5% CO₂ at 37 °C and left undisturbed for 3-5 days. Once the cells had recovered and started growing, they were passaged on a regular basis and the cell line was maintained as described below.

Maintenance

THP-1 cells were routinely grown and maintained in complete RPMI medium (with 10% NBCS) containing streptomycin-penicillin antibiotics. The cells were passaged once in three days. The cells were usually maintained in a 6-well plate. THP-1 cells were seeded at a concentration of 0.3 X 10⁶ cells per well with 3 ml of medium was added. The cells were incubated at 37 °C with 5% CO₂ until the cells grew to >80% confluence. At this point, the cells were harvested by centrifugation at 1300 rpm for 10 min at 23 °C. After washing the cells twice they were resuspended in fresh medium. The cells were seeded in a 6-well plate and allowed to grow. For infection assay experiments, when the cells were required in large number, they were grown in a 25 cm² or 75 cm² culture flasks under similar conditions.
**Frozen stock preparation**

THP-1 cells were grown to not more than 80% confluence and the cells were harvested. After washing the cells twice, they were resuspended in 1 ml of RPMI medium and the total number of cells present was counted. The cells were diluted such that 1 X 10^8 cells were present in 500 µl. This 500 µl of cells were added to a prechilled cryovial and 500 µl of RPMI with 40% NBCS and 20% DMSO was added. The cryovial was immediately transferred to a isoporpanol jacketed -1 °C cryo cooler. The cooler was kept at 4 °C for 1 h, and then transferred to -70 °C for 2-12 h. The frozen cells were then transferred to liquid nitrogen for long term storage.

**Trypan Blue Staining**

The viability of cells was determined by performing trypan blue exclusion assay as described by Shapiro (1988). Ten microliter of the cell suspension was mixed with 10 µl of 0.4% trypan blue solution in a 0.5 ml microfuge tube. The mixture was incubated for 2 min at room temperature and 10 µl of the cell suspension was placed on a haemocytometer and viewed under the microscope. The number of dead cells stained blue among the total cells was counted and the percentage viability was calculated.

**MACROPHAGE INFECTION ASSAY**

**PMA stimulation of THP-1 cells**

THP-1 cells were differentiated with phorbol-12-myristate-13-acetate (PMA) as described by Takashiba et al. (1999) with minor modifications. THP-1 cells were grown in a 75 cm^2 flask in complete RPMI with antibiotics, until the cells grew to ~80% confluence. The cells were harvested by centrifugation at 1300 rpm for 10 min at 23 °C. After washing the cells thrice with RPMI without antibiotics, they were resuspended in complete RPMI medium and counted. The cells were seeded in a 24 well plate at a concentration of 2.5 X 10^5 cells per ml per well. For each time point, triplicates were used and depending on the number of time points, as many numbers of wells were seeded identically. PMA was added to a final concentration of 400 ng/ml to each well and the cells were incubated at 37 °C with 5% CO₂ for 24 h. At the end of 24 h, the medium was removed from each well and the adhered cells were washed with RPMI to remove traces of PMA. To every well, 1 ml of complete
RPMI medium without antibiotics was added and incubated at 37 °C with 5% CO₂ for 24 h after which the cells were taken for infection assay.

**M. smegmatis culture**

A single colony of *M. smegmatis* culture was inoculated in 3 ml of BHI medium with apramycin and incubated at 37 °C at 180 rpm for overnight. Fresh overnight culture was subcultured at 1% to 50 ml of BHI broth with apramycin and incubated at 37 °C till the OD reached 0.3. This culture at 0.3 OD corresponded to 1 X 10⁷ bacteria per ml. The culture was diluted appropriately to get 2.5 X 10⁵ bacteria per 100 µl.

**Infection assay**

Infection assay was carried out based on the method described by Wieles *et al.* (1997) with slight modifications. To 100 µl suspension of bacteria containing 2.5 X 10⁵ cells in a 2 ml microfuge tube, 900 µl of RPMI medium with 10% human AB serum was added and mixed by inversion. The tubes were kept in a griener wheel and the contents were mixed well at room temperature for 30 minutes for the opsonization of bacteria to take place. At the end of 30 min, 1 ml of RPMI medium was removed from each well containing the differentiated THP-1 cells and 1 ml of RPMI medium containing the opsonized bacteria was added. Infection of the macrophages with bacteria was carried out at a multiplicity of infection of 1:1 (macrophage:bacteria) for 2 h at 37 °C with 5% CO₂. After two hours, the medium was removed, cells were washed with warm HBSS once and 1 ml of RPMI medium with streptomycin (40 µg/ml) was added to each well. The cells were incubated at 37 °C with 5% CO₂. During extended infection periods, the medium was replaced once in two days or when the pH of the medium changed, which ever was earlier.

At every time point, three wells were taken for determining the intracellular survival of *M. smegmatis* in the macrophages. At the respective time points, the medium from the well was removed and the cells were washed gently with warm HBSS, thrice. Then, 1 ml of sterile deionized water was added to the macrophages and incubated at room temperature for 3 min. The macrophages were lysed by vigorous and repeated pipetting and the 1 ml of the macrophage lysate containing the bacteria was collected in a sterile microfuge tube. Serial ten-fold dilutions were done using this 1 ml of recovered bacteria and 100 µl of every dilution was spread on a LB agar plate with
apramycin. The plates were incubated at 37 °C for 3-5 days after which the number of colony forming units was counted and the total number of surviving bacteria at every time point was calculated.

The number of bacteria surviving at every time point of infection was obtained in triplicates. The mean of the values from three independent wells were used to calculate the standard deviation and standard error. A graph was plotted with the total number of surviving bacteria (Y-axis) against the time at which the viability of *M. smegmatis* was assessed (time in hours after infection in the X-axis). The kinetics of survival of *M. smegmatis* was followed using this curve. Also, the percentage of survival of *M. smegmatis* was plotted against the time of infection in hours.

**AUTOPHOSPHORYLATION ASSAYS**

sHsp18 protein purified under non-denaturing conditions were dialyzed against the autophosphorylation reaction buffer (25 mM Tris, pH 7.0, 1 mM DTT, 1 mM EDTA) at 4 °C for 16 h. The dialyzed protein was stored at -20 °C as 200 µl aliquots.

The autophosphorylation assay for sHsp18 was carried out as described in Preneta *et al.* (2004). Two hundred microliter of sHsp18 protein (5 µg) in the reaction buffer was taken for the assay and MgCl₂ was added to a final concentration of 5 mM. The phosphorylation reaction was initiated by the addition of 10 µCi of [γ³²P] ATP and the assay was carried out at 30 °C for 60 minutes. At the end of the reaction, 4 volumes of chilled acetone was added to the reaction mix and kept on ice for 10 min. It was further incubated at -70 °C for one hour after which the precipitated proteins were pelleted by centrifugation at 12000 rpm for 15 min at 4 °C. The protein pellet was air dried for 5 min, resuspended in 1X SDS-sample buffer and separated on a 15% SDS-polyacrylamide gel. When the dye front reached the end of the gel, electrophoresis was continued for another 30 min to remove the unincorporated label from the gel. After the completion of electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane. The transfer set up was similar to that used for the regular western blotting transfer protocol. Transfer was carried out at 0.8 mA/cm² for 2 h. After the transfer, the membrane was dried briefly and kept for exposure to X-ray film at -70 °C. The signal was detected by developing the x-ray film by the standard procedure.
**Effect of detergent**

For the autophosphorylation assay in the presence of detergent, DOC was added to the protein to a final concentration of 1% (Kantorow *et al.*, 1995). The autophosphorylation reaction was then carried out as described above.

**Acid/base stability assay**

Acid-alkali stability of the sHsp18 autophosphorylation was done based on the method described by Pollack and Singer (2001). The autophosphorylation assay was carried out in triplicates as described above. The samples were loaded on the gel such that every set was separated from the other by two empty lanes and electrophoresed. The proteins were then transferred on to a nitrocellulose membrane. The membrane was cut at the empty lane position between the sample sets to get three pieces of membranes with identical set of samples. One membrane was left untreated. The second membrane was immersed in 0.2 M HCl and incubated at 50 °C for 30 min. The third membrane was soaked in 1 M NaOH and incubated at room temperature for 30 min. At the end of 30 min, all the membranes were dried completely and taken for autoradiography.

**INTRINSIC tryptophan fluorescence analysis**

Intrinsic fluorescence analysis was carried out as described by Muchowski and Clark (1998) with slight modifications. Ten microgram of the purified sHsp18 protein was taken and the volume made upto 1 ml with the respective buffer in which the protein was present. The protein was transferred to a quartz cuvette and the fluorescence was measured at an excitation of 290 nm or 295 nm. The emission was recorded as the wavelength scan was done from 250 nm to 800 nm at a scan speed of 1500 nm/min. The excitation and emission slit was set at 10 nm and the PMT voltage at 400 V.

**Image analysis**

**Analysis of 1D gels**

One-dimensional gels (SDS-polyacrylamide gels) were analyzed using ImageQuant™ (GE Healthcare) software. The gel image was first imported to the analysis module in gray scale. The analysis was performed in automatic or manual mode. The lanes were detected followed by the detection of the protein bands. The background was
then subtracted using the standard rolling ball method. On providing the concentration and molecular weight of the protein bands in the marker lane, calibration was performed and the concentration and molecular mass of the protein bands in the sample lanes were determined. The software finally generates a report with the band intensity as volume (with and without background), concentration, relative mobility and molecular mass. This report contains the above details for every protein band in every lane and can be exported in the form of an excel sheet for further analysis.

**Analysis of 2D gels**

Two dimensional gel image analysis was carried out using ImageMaster™ 2D Platinum v7.0 (IMP7) (GE Healthcare) software as per manufacturer’s instructions. The image analysis was carried out using the gel images of *E. coli* total cell lysate with (induced) and without (uninduced) overexpressed sHsp18. The gel images were first imported to the image pool. The images were cropped if necessary before starting the analysis. A new project was created in which a match set and class set was added for each set of induced and uninduced gels. The images were imported into the matchset and the spots were detected. The values for saliency and smoothness were adjusted to remove artifacts and non-protein impurities from the gel. Some spots which were improperly detected were edited (split or merged) as required. After setting landmarks (atleast ten spots for 18 cm gel image) spanning across the gel, the gels were matched. The matched gels were then imported to the class and the matching was carried out between the uninduced and induced set of gels. Analysis of the class generated a report which gives the fold change for every spot along with the statistical significance of the fold change.

**BIOINFORMATIC ANALYSIS**

**Amino acid composition**

To determine the arginine content of the *E. coli* proteins and sHsp18, the protein sequences were analyzed for the amino acid composition using BioEdit software (Hall, 1999). The protein analysis option in BioEdit determines the mol% of all the 20 amino acids present in the sequence.
**Multiple sequence alignment**

The multiple sequence alignments were performed using the ClustalW server from the EBI site - [http://www.ebi.ac.uk/Tools/clustalw2/index.html](http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007)

**Prediction of phosphorylation sites**

The following servers were used for predicting the potential phosphorylation sites in the sHsp18 sequence.

- MotifScan - [http://myhits.isb-sib.ch/cgi-bin/motif_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Obenauer et al., 2003)
- NetPhosBac - [http://www.cbs.dtu.dk/services/NetPhosBac-1.0/](http://www.cbs.dtu.dk/services/NetPhosBac-1.0/) (Miller et al., 2009)
- Phosida - [http://141.61.102.18/phosida/index.aspx](http://141.61.102.18/phosida/index.aspx) (Gnad et al., 2007)
- PPsearch - [http://www.ebi.ac.uk/Tools/ppsearch/index.html](http://www.ebi.ac.uk/Tools/ppsearch/index.html) (Sigrist et al., 2002)

**Signal Peptide prediction**

- SecretomeP 2.0 server - [http://www.cbs.dtu.dk/services/SecretomeP/](http://www.cbs.dtu.dk/services/SecretomeP/) (Bendtsen et al., 2005)