Chapter 2

# *Materials and methods*

## 2.1 Bacterial strains and plasmids

The following strains of mycobacteria and *E. coli* were used in this study.

### 2.1.1 Mycobacterial strains

| Mycobacterium species | Reference strains | source |
|---|---|---|
| *Mycobacterium asiaticum* | | CDRI |
| *M. aurum* | T15 | JALMA, Agra |
| *M. avium* | ATCC | CDRI |
| *M. bovis BCG* | BCG Japan | AIIMS, New Delhi |
| *M. fortuitum* | ATCC6841 | JALMA, Agra |
| *M. phlei* | MTCC1724 | Ranbaxy, New Delhi |
| *M. simiae* | | CDRI |
| *M. smegmatis* | $mc^2155$ | Snapper *et al.* (1990) |
| *M. tuberculosis* H37Rv | TMC102 | Treudeau Mycobacterial Culture Collection Centre |
| *M. bovis* | | IVRI, Izatnagar |

### 2.1.2 *Escherichia coli* strains

| **Strain** | **Genotype** | **Source** |
|---|---|---|
| DH5α | $F^-$Φ80d *lac* ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd* R17 ($r_k$-$m_k$+)*pho*A *sup*E44λ$^-$ *thi*-1 gyrA96 *rel*A1 | BRL |
| NM522 | F'*pro*$A^+B^+$*lac*$I^q$ Δ(*lac*Z)M15/ Δ(*lac*-*pro*AB)*gln*V *thi*-1 Δ(*hsd*S-*mcr*B)5 | NEB |
| DHM 1 | $Cya^-$ | Hybrigenics (Paris) |
| BL21(DE3) | $F^-$ *omp*T *hsd* $S_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) | Novagen |
| BL21(DE3) pLysS | $F^-$ *omp*T *hsd* $S_B$ ($r_B^-$ $m_B^-$) *gal dcm* (DE3) pLysS ($Cm^R$) | Novagen |

### 2.1.3 Plasmids

| Sr. No | Plasmid | Genotype/Character | Source/Reference |
|---|---|---|---|
| 1 | pUC19 | Amp $^{r}$ | GIBCO- BRL<br>MBI Fermentas |
| 2 | pMV206 | Km$^{r}$ , Ori M | Stover *et al.* (1991) |
| 3 | pMV261 | Km$^{r}$ , Ori M, P $_{hsp60}$ | Stover *et al.* (1991) |
| 4 | pMV306 | Km$^{r}$ ,*att*, *int*, | Stover *et al.* (1991) |
| | pMV361:Rv3878<br>(Right orientation) | Km$^{r}$ ,*att*, *int*, | This study |
| | pMV361:Rv3878<br>(Worng orientation) | Km$^{r}$ ,*att*, *int*, | This study |
| 5 | pMV361 | Km$^{r}$ ,*att*, *int*, P $_{hsp60}$ | Stover *et al.* (1991) |
| | pMV361: GFP | Km$^{r}$ ,*att*, *int*, P $_{hsp60}$ | This study |
| 6 | pET14b | Amp $^{r}$ | Novagen |
| 7 | pTOPO2.1 | Amp $^{r}$ | Invitrogen |
| 8 | pMV261: GFP | Km$^{r}$ | This study |
| 9 | pMV261: Rv3878 | Km$^{r}$ | This study |
| 10 | pMV261: Rv3878: GFP | Km$^{r}$ | This study |
| | pMV306:$P_{1500}$: GFP | Km$^{r}$ | This study |
| 15 | pDE2GFP | amp$^{r}$ | Clontech |
| 16 | pUT18C | Amp $^{r}$ | Hybrigenics(Paris) |
| 17 | pKT25 | Km$^{r}$ | Hybrigenics(Paris) |
| 18 | pUT18C:library of *M. tuberculosis* genomic DNA | Amp $^{r}$ | This study |
| 19 | pKT25: Rv3878 | Km$^{r}$ | This study |
| 20 | pUT18C- Zip | Amp $^{r}$ | Hybrigenics(Paris) |
| 21 | pKT25- Zip | Km$^{r}$ | Hybrigenics(Paris) |

## 2.2 Standard Markers

| Marker | DNA/Protein sizes (bp/kDa) | Source |
|---|---|---|
| **A. DNA fragment sized markers (bp)** | | |
| λ DNA/ *HindIII EcoRI* Double Digest | 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564,125 | MBI, Fermentas |
| 50 bp Ladder | 1000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50 | MBI, Fermentas |
| 100 bp Ladder Plus | 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 | MBI, Fermentas |
| 1 kb Ladder | 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 | MBI, Fermentas |
| **B. SDS-PAGE Protein Marker (kDa)** | | |
| SDS 7B | 185, 115, 84, 61, 55, 36, 31 | Sigma |
| Protein Ladder | 180, 130, 100, 73, 54, 40, 36, 24, 16, 10 | MBI, Fermentas |

## 2.3 Media and buffers

The composition of buffers, media, antibiotics, dyes and stains used in this investigation are listed in Appendix.

## 2.4 Chemicals and biochemicals

Molecular biology grade chemicals and general molecular biology products including antibiotics and enzymes were purchased from Sigma, USA. The restriction endonucleases, *Taq* polymerase and other DNA modifying enzymes were purchased from MBI Fermentas and New England Biolab. Laboratory chemicals, reagents for protein purification, column for His tag protein purification were purchased from Amersham Pharmacia.

Milli RO grade water (Millipore) was used for the preparation of media and general buffers. Milli Q grade RNase/DNase free water was used for preparation of reagents and solvents for protein and DNA analysis.

## 2.5 Primers

### 2.5.1 Primers for Rv3878

1. 3878F -5'-CGGGATCCCGCAATGGCTGAACCGTTGGC 3'
2. 3878RS- 5'-CGGGATCCCGCTACAACGTTGTGGTTGTTG-3'
3. 3878 R – 5' CGGGATCCCGCAACGTTGTGGTTGTTGAGG-3'

### 2.5.2 M 13 standard primers

1. M13F- 5'-GTAAAACGACGGCCAG-3'
2. M13R- 5'-CAGGAAACAGCTATGAC-3'

### 2.5.3Primers used for sequencing *M. tuberculosis* genomic library clones

BTHF1 – 5'-TGC AGG TCG ACT CTA GAG-3'

## 2.6 General methods

### 2.6.1 Staining of acid fast bacilli

A loopful of mycobacterial culture was emulsified in saline on a clean glass slide, air-dried and heat fixed. The smear was stained using carbol fuchsin and heated for 5-7 min till vapours appeared. The slide was cooled and excess stain was washed using tap water and decolourized using acid-alcohol mixture for 30 sec. The slide was washed and counterstained using malachite green for 2 min. Excess stain was washed off, the slide was air-dried and the smear was observed under oil immersion objective. Mycobacterial cells are seen as pink rod shaped structure while the background and contaminating organisms stain green.

### 2.6.2 Isolation of genomic DNA from mycobacteria (Connell, 1995)

Mycobacteria were inoculated on LJ slants and incubated at 37 $^0$C for clear luxuriant growth to occur (1-2 weeks for fast growers, 3-4 weeks for slow growing organisms). The cells were harvested by scraping the growth using an inoculation loop and suspending in TES buffer. The cells were inactivated at 80$^0$C for 1 hr. Lysozyme was added (1 mg/ml final concentration) and the mixture was incubated overnight at 37 $^0$C. On incubation, SDS (final concentration 100 μg/ml) was added, cells vortexed and incubated at 50 $^0$C for 1 hr. Equal

volume of buffered phenol was added, mixed properly and the phases separated by centrifugation at 10,000 g for 15 min at 4 $^0$C. DNA from the aqueous phase after centrifugation was precipitated using equal volume of chilled isopropanol and incubated over ice for 30 min. The precipitated DNA was pelleted by centrifugation, washed twice with 70% and once with 96% ethanol, air dried, resuspended in TE buffer, and stored at – 20 $^0$C.

### 2.6.3 Agarose gel electrophoresis (Sambrook *et al.*, 1989)

The gel electrophoresis of DNA was carried out using horizontal submarine gel electrophoresis. Powdered agarose was weighed and suspended in 1X TBE/ TAE buffer, melted by boiling and cooled to 50$^0$C. The cooled agarose was poured into a casting tray fitted with a comb and the gel allowed to solidify for 30-40 min. The casting tray was then placed in an electrophoresis apparatus filled with 1X buffer. The DNA sample to be analyzed were mixed with 6X tracking dye (final concentration 1X ) and loaded into the wells. Electrophoresis was carried out at a constant voltage of 5-10V/cm. After the required amount of separation, the DNA was stained using ethidium bromide and visualized under UV light. In cases where the DNA bands to be eluted, preparative agarose were prepared using either routine or low melting point agarose.

### 2.6.4 Dephosphorylation of plasmid DNA (Sambrook *et al.* 1989)

The vectors utilized for cloning and subcloning were dephosphorylated using calf intestinal alkaline phosphatase (CIAP). The dephosphorylation was carried out in the same restriction digestion reaction mixture. Alternatively, purified fragments were dephosphorylated in CIAP buffer. One unit of CIAP was added to 0.15 pM of ends and incubated at 37 $^0$C for 30 min, followed by addition of another 1 unit and a further incubation step of 30 min at 37$^0$C. The mixture was extracted with phenol and then choloroform- isoamyl alcohol. The aqueous phase was mixed with sodium acetate (0.3M final concentration) and equal volume of isopropanol to precipitate the DNA. DNA was pelleted, washed, and suspended in Milli Q water.

### 2.6.5 Transformation of *E. coli* (Hanahan, 1983)

An overnight culture of *E. coli* DH5α was set up in LB broth and incubated at 37 $^0$C under shaking conditions. The seed culture was inoculated at 1:50 dilution in fresh LB and incubated at 37 $^0$C under shaking condition for 3-4 hr. The culture was centrifuged at 5000 g for 15 min at 4 $^0$C to pellet the cells and the supernatant was discarded. The pellet was resuspended in chilled TFB I buffer and centrifuged. The cells were resuspended in TFB II buffer and incubated over ice for 30 min. The cells were pelleted by centrifugation and the cell pellet resuspended in about 1/12.5$^{th}$ volume of fresh TFB II. The cells were aliquoted and kept over ice. DNA was added to the tubes and the mixture incubated over ice for another 30 min. Heat shock was given in a water bath set at 43.5 $^0$C for 45 seconds. About 6.5 volumes of Z broth was added and the mixture incubated at 37 $^0$C for 40 min. Transformation mixture was plated on selective media. The plates were incubated at the required temperature.

### 2.6.6 Electroporation of plasmids into mycobacteria (Cornell, 1995)

Mycobacteria were inoculated in LB containing glycerol and Tween 80 or Sauton's media and incubated at 37$^0$C under stationary or shaking conditions for the required time. The cells were pelleted at 5000 g for 10 min at 4$^0$C. The supernatant was discarded and the cells resuspended in chilled 10% glycerol. Again, the cells were pelleted and washed twice with chilled 10% glycerol,centrifuged and supernatant discarded. The cells were finally resuspended in 1/50$^{th}$ the original volume, in 10% glycerol, and aliquoted in 0.4 ml into separate eppendorf tubes. DNA was added to the tubes and the mixture incubated over ice for 5 min. Electroporation cuvettes (0.2 cm gap) were loaded and the cells pulsed at 2.5 KV, 25 μF and 1000 Ω. The pulsed cells were immediately kept over ice and then transferred to 2.5 volumes of SOC medium.The cells were incubated at the required temperature and time (3-4 hr for fast growers, overnight for the rest ) and then plated on selective media.

### 2.6.7 Restriction endonucleases digestion (Sambrook *et al.*, 1989)

DNA was digested with appropriate restriction enzymes in buffers recommended by manufacturers. One microgram of DNA was digested in a total reaction volume of 30 μl for 16 hr at the optimal temperature for the enzyme(s). The reaction was terminated by heating

analyzed by agarose gel electrophoresis using proper controls and molecular size markers.

### 2.6.8 Ligation

(i) Self ligation

| | |
|---|---|
| DNA | 10-100 ng |
| 10X buffer (T4 DNA ligase ) | 2.0 µl |
| 10mM ATP | 2.0 µl |
| Milli Q $H_2O$ to a total volume of | 20 µl |

T4 DNA ligase (1-3 units ) added and incubated overnight at 16 $^0$C.

(ii) Cloning of insert (staggered ends )

Insert to vector ratio was more than 2:1

| | |
|---|---|
| Insert DNA | 0.2- 1 µg |
| plasmid DNA | 0.1- 0.5 µg |
| 10X T4 DNA ligase buffer | 2.0 µl |
| 10 mM ATP | 2.0 µl |
| Milli Q to a total volume of | 20 µl |

T4 DNA ligase (1-2 units) was added, mixed and incubated at 16$^0$C overnight.
Ligase was heat inactivated at 65 $^0$C for 15 min before transformation.

### 2.6.9 Nucleotide sequencing of cloned inserts

The plasmid used as template for sequencing was isolated using Qiagen plamid mini kit (Qiagen, Germany) as per instructions. Cells from 5 ml of overnight culture of recombinant *E. coli* in LB containing antibiotic were harvested by centrifugation and the pellet resuspended in 250 µl of buffer P1. After mixing thoroughly equal volume of buffer P2 was added, mixed gently and incubated at RT for 5 min. Buffer P3 (350 µl) was added and incubated on ice for 15 min .The debris was removed by centrifugation twice at 20000 g for 30 min at 4$^0$C . The clear supernatant was added to Qiagen spin column and run at 6000 g. Flow through was discarded and 450 µl of buffer QBT was passed through the column. Column was again washed with buffer PE (500µl). To remove remnant of PE buffer column

was blank run and DNA was finally eluted in 30 µl elution buffer. The amount of plasmid was checked by agarose gel electrophoresis.

The sequencing reaction contained in a final volume of 10 µl:

| | |
|---|---|
| Template DNA | 10 ng |
| Primer | 20 ng |
| Big dye terminator reaction mix | 4µl |

The reaction mixture was mixed and cycled as follows.

| | | |
|---|---|---|
| 94 $^0$C | 3 min | 1 cycle |
| 94$^0$C | 20 sec | 30 cycle |
| 59 $^0$C | 20 sec | |
| 60 $^0$C | 4 min. | |
| 4 $^0$C | hold | |

The amplified product was precipitated using 0.1 volume of 3 M sodium acetate and 2.5 volumes of 96 % ethanol. The DNA was pelleted immediately by centrifugation at 20000g for 30 min, washed once with 100 µl of 70% ethanol, air- dried and suspended in 20 µl of TSR (Template suppressor reagent).

The sample was denatured at 94 $^0$C for 3 min, snap frozen on ice and transferred to 0.5 ml sample vials with rubber closure. The sample tubes were placed in the sample tray and loaded into ABI prism 310 Genetic Analyzer for sequencing.

### 2.6.10 Protein estimation (Lowry *et al.*, 1951)

The amount of protein was measured by Foline phenol reagent. The sample was taken in test tube and volume was adjusted to 300 µl with distilled water. 1 ml of solution C was added and incubated at room temperature for 15 min. 100 µl of solution D was added to the mixture and the tubes were incubated at room temperature for 45 min in dark. Absorption of the blue coloured complex was measured at 650 nm using spectrophotometer (Beckman). Amount of protein present in the experimental sample was determined from the linear graph (obtained by Beckman software) of the known concentration of the standard sample (BSA) processed in parallel with experimental sample.

### 2.6.11 Methods in protein expression and purification

**(a) Protein induction**

An isolated colony was picked up from a freshly streaked plate and inoculated in LB medium containing appropriate antibiotic. The seed culture was allowed to grow till an $OD_{600}$ of 0.8- 1 at 37 $^0$C under shaking for overnight. Fifty-fold dilution of seed culture was made in fresh LB (pH 7.2 ) containing antibiotic and incubated till an $OD_{600}$ of 0.3-0.4 . The culture was induced by addition of 1 mM IPTG and further incubated at 37 $^0$C for 3-4 hr. The cells were harvested by centrifugation and suspended in 10 mM Tris- HCl pH 8.0. The cells were stored at -20$^0$C for further analysis.

**(b) Sample preparation for protein profile analysis**

The induction of the protein was analyzed by partitioning the samples into subcellular fractions.

(i)**Total cell protein sample**: An aliquot of induced cells was taken and mixed with equal volume of 2X Laemmli sample buffer. The mixture was heated for 5 min. in boiling water bath and centrifuged at 12000g for 5 min. The supernatnant was used for analysis by denatured polyacrylamide gel electrophoresis (SDS-PAGE)

(ii) **Soluble cytoplasmic fraction:** An aliquot of induced cells was taken and disrupted by lysozyme treatment (100 µl/ml ) followed by sonication using microtip with power level set between 4-5, at 40-50 duty cycles for 15-20 bursts . The disrupted cells were centrifuged at 12000 g for 10 min.

(iii)**Insoluble fraction:** The pellet obtained after removal of soluble cytoplasmic fraction, was resuspended in 1% SDS with heating and vigourous mixing or sonication.

All the above fractions were were analyzed by SDS-PAGE, after mixing with equal volume of 2X Laemmli sample buffer. The mixture was heated for 5 min, in boiling water bath and SDS-PAGE was performed.

### 2.6.12 Denatured SDS- polyacrylamide gel electrophoresis

The vertical slab gel unit was assembled according to the manufacturer's instructions (Bio-Rad or BRL). The resolving monomer solution of desired percentage of acrylamide was prepared in Tris 0.375 M (pH 8.8) containing 0.1% SDS, 0.05% APS and 0.05% TEMED. The gel was cast by introducing the monomer solution into the gel mold in a steady stream and overlaid using water or water-saturated butanol solution. Polymerization was allowed to occur at RT. The stacking monomer solution was made of 3.5% acrylamide in 0.125 M Tris, pH 6.8 containing 0.1% SDS, 0.05% APS and 0.01% TEMED. The gel was cast by introducing the monomer solution on top of the polymerized resolving gel. Appropriate size combs were introduced at the top and staking gel allowed to polymerize at RT, at least for 90 min prior to use.

After polymerization the comb was carefully removed and the sandwich containing SDS-PAGE was fixed with buffer unit of the apparatus. The upper and lower chambers of the tank buffer were filled with Tris-Glycine buffer and the samples were loaded after cleaning the wells with the tank buffer. The gel was electrophoresed initially at 80 V till the samples entered the resolving gel and then continued till the end at 120 V. The gel was removed from the apparatus and stained with coomassie brilliant blue R-250 solution for 1 hr at 65 $^{0}$C and destained with gentle shaking. The gel bands were analyzed along with appropriate protein molecular weight markers and documented by gel documentation system (UVP) and stored for future reference in gel storage solution.

### 2.6.13 Optimizing expression and solubilization of protein

The protein induction conditions were optimized so as to maximize the protein in soluble fraction. The parameters that were varied are:

(i) *E. coli* host strains: BL21(DE3), BL21(DE3) pLyS,

(ii) IPTG concentration for induction : 0-1000 μm

(iii) Temperature at which incubated after induction: 18-37 $^{0}$C

(iv) Time of incubation after induction : 2-20 hr

### 2.6.14 Large scale purification of recombinant protein using His Bind resin

The recombinant fusion protein was purified in quantities of 20 mg /batch using His Bind resin (Amersham). All the steps were carried out at $4^0$C. The cells were harvested from induced cultures and suspended in binding buffer (200 mg cells /ml). The cells were disrupted by sonication and the cell debris was removed by centrifugation at 12000 g for 20 min. A 5 ml HR $Ni^+$ column (30 mg /ml binding capacity) and charged with 10 volumes of Milli Q water and equilibrated with 5 volumes of binding buffer was used. The supernatant containing soluble proteins was loaded onto the column near its full capacity. The column was washed with 10 volumes of binding buffer and 5 volumes of wash buffer. The protein was eluted using elution buffer. The column was resused after striping with strip buffer and washing with Milli Q water.

### 2.6.15 Western blot analysis (Harlow and Lane, 1989)

The western blot were prepared by transferring the separated proteins from the polyacrylamide gel onto a nitrocellulose membrane in a Trans blot apparatus ( Bio- Rad Min.i protein III ) as described by Towbin *et al.*(1979). After electrophoresis the gel was soaked in transfer buffer. The gel was sandwiched between whatman paper No. 3 on one side and nitrocellulose membrane (Sigma) on the other side. Additional sheets of Whatman paper No. 3 were added on both sides and fixed tightly in the Trans-blot apparatus. Care was taken to avoid any air bubbles to trap between gel and nitrocellulose membrane. The apparatus was placed in such a way that the nitrocellulose membrane faces the anode. The transfer was carried out at 60 V for 2 hr at 4 $^0$C. Complete transfer of protein onto the membrane was checked visually by transfer of the coloured protein marker. Blots after transfer were washed with transfer buffer and blocked with 0.5% Tween 20 in TBS solution for overnight at $4^0$C. Blots were transferred in primary antibody solution (1:500 to 1:5000 dilutions) and incubated for 2 hr with continuous shaking on a rockmat. The blots were washed three times with 0.05% Tween in TBS (washing solution) for 10 min each. Goat anti- rabbit IgG (Promega) conjugated with HRP, were diluted (1:2500) in 0.05% Tween 20 (in TBS). The blots were subsequently washed with washing solution for 10 min with continuous shaking. Blots were developed by peroxidase substrate DAB, washed with distilled water and stored.

### 2.6.16 Generation of antibody against recombinant protein (Harlow and Lane, 1989)

To raise polyclonal antibody against recombinant protein, rabbits were immunized subcutaneously with 200 μg purified protein, emulsified in Freund's incomplete Adjuvant (Sigma). At 21 days interval from first immunization, rabbits were given two booster dose of 100 μg protein emulsified in Freund's incomplete adjuvant. Rabbits were bled after 21 days of second booster dose, and the isolated serum was stored at -20$^0$C after adding 0.02 % $NaN_3$.

### 2.6.17 ELISA (Harlow and Lane, 1989)

The antibody titer in the patient serum was analysed by Enzyme linked immuno sorbant assay (ELISA) using HRP linked secondary antibodies and ortho phenylene diamine (OPD) as the chromogenic substrate. Purified antigen ( Rv3878 ) was coated on the ELISA plates in carbonate buffer at 5 μg/ml concentration overnight at 4 $^0$C in humidified chamber and blocked with blocking buffer (5 % milk in TBS ) for 2 hr at 37$^0$C. Primary antibody dilutions were made (1:25- 1:50 time in 1 % skim milk) and incubated for 1 hr at 37$^0$C followed by 6 washes with TBST . HRP conjugated secondary antibodies were diluted at 1:2500 in 1% skim milk and added to the well and further incubated for another 1 h. After 6 washes with TBST, plates were developed for 2 min by adding OPD solution at 1 mg/ml concentration in citrate buffer (pH 5.5) with trace of $H_2O_2$ as catalyst. Reaction was stopped by adding 7.5% $H_2SO_4$ and read at 492 nm in ELISA reader.

### 2.6.18 Southern hybridization (Southern, 1975)

#### 2.6.18.1 Transfer of DNA from gel onto nitrocellulose membrane

The DNA to be transferred was electroporated and transferred onto the nitrocellulose membrane (Whatman) by capillary transfer (Bio Rad ). The gel was treated with 4 volumes of denaturation buffer with gentle agitation for 30 min followed by treatment with 4 volumes of neutralizing buffer for 30 min and gentle shaking. DNA was transferred from gel onto nitrocellulose membrane (NC) in 10 X SSC for overnight. After the transfer, the blotting sheets were removed and the membrane was marked with pen for orientation and rinsed in 10X SSC for 5 min.

### 2.6.18.2 Crosslinking

NC membrane containing DNA were placed on top of the presoaked (10X SSC)

3 mm Whatman paper, linked by UV Cross linker ( Stratalinker- Stratagene ) for 50 seconds at an energy of 120,000 microjoules/$cm^2$ .

### 2.6.18.3 Preparation of DIG-labeled probes

Linear template DNA was labeled with DIG-DNA labeling kit (Boehringer Mannheim) and used as a probe. The DNA template was denatured in a boiling water bath for 5 min and quickly chilled on ice.

The labeling mixture consisted of the followings:

| | |
|---|---|
| DNA template (1-3 μg) in Milli Q | 15 μl |
| Hexanucleotide mixture (10X) | 2 μl |
| dNTP labeling mixture (10X) | 2 μl |
| Klenow enzyme | 2 units |

The labeling was done by incubation at 37 $^0$C for 60 min. After labeling the mixture was directly used for hybridization experiment.

### 2.6.18.4 Prehybridization

Prehybridization and hybridization were done in the rotary oven ( Amersham Life Science). Cross linked blots were placed in hybridization bottle(s) containing 20 ml prehybridization solution per 100 $cm^2$ of membrane surface area. Prehybridization was carried out at 67 $^0$C for 2-3 hr.

### 2.6.18.5 Hybridization

After prehybridization the solution was replaced with the hybridization solution.
Hybridization was carried out at 67.8$^0$C for 16 hr, and the blots were washed as follows:

2 × 5 min at room temperature on a rockmat with 2X SSC and 0.1% SDS
2 × 15 min at 67.8$^0$C with 0.1 SSC and 0.1% SDS with shaking.

### 2.6.18.6 Detection of bound DIG- labeled probe

The blot after washing was equilibrated in GBI for 1 min. The membrane was blocked by using GBII buffer for 1hr with shaking. After blocking, the solution was replaced with anti-DIG alkaline phosphatase labeled antibodies in GB2 buffer ( 1:7500 dilultion) and incubated for 30 min with shaking. After incubation, membranes were washed with ample amount of GBI buffer thrice for 10 min each. The membranes were equilibrated in GBIII buffer for 2-3 min and incubated in developing solution. The membranes were monitored till the band appeared and were washed extensively with distilled water and kept for documentation and photography.

### 2.6.19 Immuno- fluorescence microscopy (Pogliano *et al.*, 1995)

Slides were coated for 2 min in 1 % polyethylenemine or for 1 hr at room temperature in 0.01 % poly (L- lysine). Coated slides were washed with PBS and air dried. Log phase grown bacteria were centrifuged and washed two times with PBS. Finally bacteria were suspended in PBS. Aliquots of bacterial cells were allowed to adhere on poly-L-lysine coated glass coverslips. Excess cells were removed and subsequently washed with PBS. Cells were fixed with fixative 2% paraformaldehyde in PBS (pH 7.2 ) for 30 min at room temperature or 80% methanol for 1 min. Fixed cells were subsequently washed with PBS. After washing with PBS, excess aldehyde was quenched by adding 0.05% glycine (w/v) in PBS for 10 min at room temperature. For observation of GFP, fluorescent cells can be mounted at this stage. For immunolocalization, cells were further permeabilized and labeled with specific antibody. Cells were permeabilized with permeabilizaton buffer (0.1% TritonX -100,10 mM EDTA, 0.05% Glycine) , twice for 2-5 min each. To reduce nonspecific interactions and background fluorescence, adhered cells on slide were blocked in blocking buffer ( 0.1-0.5 % acetylated BSA in PBS and kept for half an hr at room temperature or overnight at 4 $^{0}$C for blocking. Cells were further treated with primary antibody (1:100 -1:500 dilution in blocking buffer) for 4 hr at 4 $^{0}$C. After washing with blocking buffer (6 times) cells were incubated with secondary antibody anti rabbit IgG (1:50 times) labeled with florescent dye at 4 $^{0}$C for 4 hr in dark .Secondary antibodies were aspirated and washed with blocking buffer ( 6 times ). Cells were finally mounted in mounting medium (90% glycerol alone or containing nucleic acid staining dye DAPI or

hoescht (Molecular Probe ) and stored for analysis at $4^0$C in the dark. Images were collected using 60X 1.4 NA Plane Apochromate lens on Bio-RAD Radiance 2100 Confocal microscope attached with a Nikon fluorescence microscope.

### 2.6.20 Localization of Rv3878

#### (a) Osmotic shock to recombinant cells expressing Rv3878 in fusion with GFP (Hosam *et al.*, 2005)

Cells were grown at 37 $^0$C until OD $_{600}$ reached around 1.5. For osmotic lysis 1 ml culture was taken and centrifuged to remove growth medium. Pellet was washed thrice in 20 mM Tris-HCl (pH 7.5). Cells were suspended in hypertonic solution containing 20 mM Tris-HCl (pH 7.5), 20 % sucrose, and 0.5 mM EDTA and incubated in ice for 10 min. After incubation on ice cells were pelleted by centrifugation at 12000 g for 10 min at 4 $^0$C. Pellet was suspended in 200 ul of hypotonic solution and incubated in ice for additional 10 min.

For visualization of GFP protein, cells were labeled with fluorescent dye FM4- 64 (Molecular Probe). Cells were washed with PBS and fixed with 2% paraformaldehyde at room temperature for 10 min. To remove formaldehyde cells were washed and suspended in PBS and mounted on slide with mounting medium containing antifade PPD (paraphenyle diamine) reagent. Cells were visualized under Nikon fluorescence microscope.

#### (b) fluorescence microscopy

Mycobacteria grown in 10 ml Sauton's medium upto mid log phase were pelleted and washed three times with TTBS ( 0.05 % Tween 20). Pellets were suspended in TTBS and incubated in primary antibody (100- 500 dilution) against Rv3878 at 37 $^0$C for 1 hr with gentle shaking. Cells were washed three time with TTBS and again incubated with secondary antibody labeled with FITC for 1 hr in dark with gentle shaking. Cells were washed three times with TTBS. For analysis under fluorescence microscope cells were fixed by paraformaldehyde and then washed with TTBS. Cell was labeled with membrane staining dye FM 4-64. Finally cell were mounted with mounting medium containing antifade reagent PPD.

### 2.6.21 Preparation of *M. tuberculosis* subfractions ( Lee *et al.*, 1992)

*M. tuberculosis* cells were grown to late log phase (14 days) in 200 ml sauton's medium. Cells were heat inactivated at $80^0$C for 1 hr and harvested by centrifugation. Pellet was washed two times with chilled PBS and finally suspended in PBS. Cells were broken by sonicatior at $4^0$C. Unbroken cells were removed by centrifugation and supernatant was kept for further application. Aliquots of supernatant were kept as total fraction. For isolation of cell wall fraction supernatant was centrifuged at 20000 g for 1 hr. Pellet was kept as a cell wall fraction and supernatant was mixture of cell membrane and cytosolic fraction. To isolate membrane fraction, supernatant was spin in ultracentrifuge at 100000 g for 4 hr. Pellet was kept as a cell membrane fraction. Supernatant was kept as a cytosolic fraction. Proteins were estimated in different fractions.

### 2.6.22 Generation of *M. tuberculosis* genomic library

A *M. tuberculosis* genomic library was prepared in plasmid pUT18C encoding N-terminal adenylate cyclase. *M. tuberculosis* genomic DNA was digestesd. DNA was digested with *Sau*3AI. Digested product was resolved in agarose gel with DNA molecular weight markers. Fragment size ranging between 0.2-2 kb was isolated from the gel. Isolated fragments were CIAP treated and then purified by alcohol precipitation methods. CIAP treated fragments were ligated with vector pUT18C, linearized with *BamH*I. Ligation product was transformed in *E. coli* DH5α. Clones were selected on LB+amp plate. Approximately 12000 individual clones were collected. Individual clones were pooled in LB broth and plasmids were isolated. Plasmids were stored as *Sau3A*I library. To check the efficiency of the library plasmids were isolated from 50 individual clones and digested with the *Xba*I and *EcoR*I and inserts were examined by agarose gel electrophoresis. More than 70% clones had inserts of varying sizes.

### 2.6.23 BATCH complementation assay (Karimova *et al.*, 1998)

Efficiency of interaction between different hybrid clones was quantified by measurement of ß- galactosidase activity in liquid culture. For measurement bacteria were grown in LB broth in the presence of 0.5 mM IPTG and appropriate antibiotics at 30 $^0$C for 14 to 16 hr. First, cells were diluted 1:5 in M63 medium and optical density $OD_{600}$ was

recorded. Cells were then permeablized by 35 µl of toluene and 35 µl of 0.1% SDS in 2.5 ml of bacterial suspension. The tubes were vortexed for 10 sec and incubated at 37 $^0$C for 30 to 40 min for evaporation of toluene. For enzymatic reaction, aliquots (0.1 to 0.5 ml) of permeablized cells were added to buffer PM2 (70 mM $Na2HPO_4 \cdot 12H_2O$. 30mM $NaHPO_4 \cdot H_2O$, 1 mM $MgSO_4$, and 0.2 mM$MnSO_4$, pH 7.0), containing 100 mM ß- mercaptoethanol, to a final volume of 1 ml. The tubes were incubated at 28 $^0$C in a water bath for 5 min. The reaction was started by adding 0.25 ml of 0.4% 0-nitrophenol-ß-galactoside (ONPG) in PM2 buffer (without ß- mercaptoethanol). The reaction was stopped by adding 0.5 ml of 1 M $Na_2CO_3$ solution. $OD_{420}$ was the recorded. The enzymatic activity *A* (in unit per miller) was calculated according to the following equation *A* =100 X ($OD_{420}$ of the culture/time x V x $OD_{600}$).

### 2.6.24 Assessment of cell surface hydrophobicity by Congo red binding assay (Etienne *et al.*, 2002)

Congo red binding assay was performed to measure hydrophobicity of recombinant *M. smegmatis* . Bacteria was cultured for three days in 5 ml MB7H9 broth supplemented with Congo red (100 ug/ml) and kanamycin (25 ug/ml). Cells were recovered from cultured broth, washed with PBS to eliminate unbound Congo red completely and then resuspended in 1 ml acetone. After gentle shaking for 2 hr, cells were pelleted by centrifugation and the concentration of Congo red released from bacteria into supernatant was measured by spectrophotometer at $OD_{488}$ nm. The relative binding index was defined as the $OD_{488}$ of acetone extract divided by $OD_{600}$ of the cell pellet.

### 2.6.25 Assessment of cell surface hydrophobicity by adherence of bacteria with xylene (Miyamoto *et al.*, 2004)

The hydrophobicity of bacteria was assessed by measuring the adherence of bacteria to hydrocarbon xylene. Briefly cells were grown upto mid log phase and then washed twice with PBS. Finally cells were suspended in PBS and adjusted to 1 at $OD_{600}$. 2 ml culture was taken from each test sample in different test tubes and 0.5 ml of xylene was added in each tube. Cells were mixed by vortex for 2 min. After standing at room temperature for 20 min, the absorbance $OD_{600}$ of lower aqueous phase was measured. The hydrophobicity of bacteria

was reflected by reduction in absorbance. Hydrophobic bacteria are assumed to remain in upper phase because of adhesion to hydrocarbons.

### 2.6.26 Autoaggregation of mycobacteria by sedimentation assay (Sherlock *et al* ., 2004)

In order to monitor differences in autoaggregation, an assay to monitor bacterial settling kinetics over time was carried out. *M. bovis* BCG contaning pMV261 :GFP and pMV261 :Rv3878:GFP were grown upto mid log phase in MB 7H9 ADC containing kanamycin . The culture were pelleted and resuspended in MB7H9 medium and OD of the two cultures was approximately equalized. At regular time intervals, 200 ul of culture was taken and transferred in a microtiter plate incubated and pre-cooled on ice . At the end of the experiment $OD_{600}$ nm were recorded by ELISA reader.

### 2.6.27 Isolation of total lipids and glycopeptidolipids (GPLs ) (Patrick *et al.*, 1978)

Recombinant strains of *M. smegmatis* (pMV261:GFP) and *M. smegmatis* (pMV261: Rv3878: GFP) were grown in LB broth at 37 $^0$C in presence of kanamicin and harvested ) in mid log phase by centrifugation (3000 g). Cells were washed three times in PBS and dried in freeze drier. For extraction of total lipids choloroform – methanol ( 2:1 vol/vol ; 32µl/ mg of bacteria ) was added to the dry bacteria in culture tubes with screw caps. The cells were subjected to intermittent sonication for 5 sec at every 5 hr during incubation and kept at 50 $^0$C for 18 hr. Tubes were centrifuged at 1500 g for 30 min, followed by storage of the clear supernatant at 4 $^0$C in tubes tightly closed with Teflonlined caps.

For isolation of GPLs, the lipid extract was subjected to alkaline methanolysis (0.2 M NaOH in methanol, 2 h, 40 $^0$C) to cleave esterlinked fatty acids. The mixture was neutralized with glacial acetic acid. After neutralization, samples were dried with a stream of nitrogen at 37 $^0$C. For isolation of GPLs, chloroform-methanol (2:1) followed by water was added to the dry residues, which was vortexed, mixed and centrifuged (1500 g). The upper aqueous phase was discarded and lower chloroform phase was dried in a stream of nitrogen. The contents were redissolved in chloroform- methanol (2:1 vol/vol) before chromatography.

### 2.6.28 Thin layer chromatography (Patrick *et al.*, 1978)

Total lipids and GPLs were analyzed by thin layer chromatography (TLC) on silica gel 60 plates (Merck) using different polar systems. Lipids were localized by spraying with 10% $H_2SO_4$ and subsequent charring at 110 $^0$C.

### 2.6.29 GFP transcriptional fusion assay during growth cycle (Siobhan *et al.*, 2001)

GFP fluorescence of bacterial culture harboring different constructs were quantified using a spectrofluorometer ( BMG Germany). Culture of different clones were grown to mid log phase in MB7H9 in a shaker at 37 $^0$C. The seed culture was inoculated in triplicate in 250 ml flask containg 50 ml MB7H9 broth. At different time intervals 100 ul culture was taken from each flask, diluted in 2 ml PBS and relative fluorescence unit (RFU) was measured using a 490 excitation filter and 520 emission filter. $OD_{600}$ nm of each culture was recorded in a spectrophotometer (LKB). Background fluorescence was minimized by subtracting the autofluorescent value of the bacteria grown in the same condition, harbouring pMV306 vector alone.

### 2.6.30 GFP translational fusion assay in different stress conditions (Siobhan *et al.*, 2002)

*M. smegmatis* containing pMV306:*hsp*$_{60}$:GFP, pMV306:*P*$_{1500}$:GFP were grown in some defined conditions to check the promoter expression in different conditions. Cultures were exposed to oxidative stress (10 mM $H_2O_2$), acid stress (pH 4.5), and heat shock (incubated at 45 $^0$C). Experiment starts with initial OD at 600 nm of 0.5 and then exposed for at least 4 hr in different stress conditions. After exposure, samples were taken out and fluorescence was measured in spectrofluorometer and optical density was taken at 600nm in spectrophotometer.

### 2.6.31 GFP transcriptional fusion assay in acidic condition (Betts *et al.*, 2002)

For the expression analysis of Rv3878 gene in acidic condition *M. smegmatis* containing pMV306:*hsp*$_{60}$:GFP and pMV306:*P*$_{1500}$:GFP were grown in different pH ranging from 4.0 to 7.0. Cells were initially grown in MB7H9 medium at pH 7.0 and then diluted in MB7H9 medium adjusted to different pH and incubated for 4 hr.

### 2.6.32 GFP transcriptional fusion assay in nutrient starvation model (Betts *et al.*, 2002)

*M. smegmatis* harbouring different promoter constructs were subjected to nutrient starvation model as described by (Betts *et al.*, 2002). Initially, bacteria were grown to OD 0.5 at 600 nm in HdeB medium supplemented with 0.08% glycerol. The cells were then diluted in different starvation medium, the standing OD being 0.05. The carbon starvation condition was created into 250 ml flasks containing 50 ml HdeB supplemented with 0.08% (vol/vol) glycerol.

For nitrogen starvation condition, $(NH_2)SO_4$ concentration in the HdeB was reduced 100 fold to 0.15 mM and 0.2% glycerol (vol/vol) . In phosphorous starvation condition phosphorous concentration was reduced 100 fold to 0.16mm as compared to control with 16 mM. Fluorescence and OD were measured in spectrofluorometer and spectrophotometer. All experiments were performed in triplicate.

### 2.6.33 Expression analysis in ex-vivo condition (Roy *et al.*, 2002)

Mouse macrophage cell line J774A was grown in RPMI 1640-10% FCS medium in 75 $cm^2$ tissue culture flasks for 3 days before the experiment. One day before experiment cells were trypsinized and washed with incomplete RPMI-1640. Cells were counted by haemocytometer and diluted in RPMI-1640- 10% FCS medium to a density of $2X10^5$ cells per ml. J774A cells (1ml/per well) were incubated overnight under 5% $CO_2$ at 37 $^0C$. Adherent monolayers were infected with gfp and promoter constructs of *M. bovis* BCG at MOI of 1-5. The 24 well plates were incubated for 4 h at 37 $^0C$ in a 5 % $CO_2$ incubator. The infected monolayers were then washed once with warm HBSS and treated with RPMI 1640-10% FCS. At different time interval, cells were taken, fixed in 2% paraformaldehyde for 10 min at room temperature and FACS analysis was done.

### 2.6.34 Circular Dichroism measurements

CD spectra were taken with a Jasco J810 spectropolarimeter. Chamber was filled up with nitrogen gas to clean oxygen out of chamber due to absorbance effect of ambient gases and most important ozone formation caused by the Xe bulbs which can kill the silver optics. The CD spectra were measured at Far UV (190-250 nm) at 20 $^0C$. Each spectrum is the average of three scans. The value obtained were normalized by subtracting the baseline

recorded for the buffer having the same concentration as in the protein. The results are expressed as relative ellipticity [θ], which is defined as [θ] = 100 x $\theta_{obs}$ / l C, where $\theta_{obs}$ is the observed ellipticity in degrees, C is the concentration of mols of residue per litre and l is the path-length through which light passes, through sample.

### 2.6.35 The thermal denaturation of protein Rv 3878

The thermal denaturation of protein Rv 3878 was carried out by measuring changes in the CD ellipticity. Protein was preincubated in presence of 5 mM SDS and 20% TFE. The samples were heated from 20°C to 100°C at the rate of 1°C/min using a programmable thermal control unit.

### 2.6.36 Chemically induced unfolding of protein Rv3878

Chemically induced denaturation of protein Rv3878 by GuHCl was performed in presence of SDS. Protein samples were incubated at different concentration of denaturant for approximately 24 h at 25 $^{0}$C to attain equilibrium. The change in structural and functional properties of protein Rv 3878, in presence of SDS was monitored by measuring optical properties with CD.

### 2.6.37 Chemical cross linking of Rv3878 protein (Banerjee *et al.*, 2005 )

Rv3878 protein was dialyzed in 20 mM phosphate buffer. Different concentrations of glutaraldehyde was incubated with 20 µg of protein for 30 min at room temperature in 20 µl of 1X cross linking buffer. Reaction was stopped by adding 1 µl of 1M Tris-Glycine buffer. Proteins were run on SDS-PAGE with molecular weight markers.

### 2.6.38 Tyrosine fluorescence measurement

Fluorescence spectra were recorded with Perkin-Elmer LS 50B spectroluminescence meter in a 5 mm path length quartz cell. Rv3878 protein in 20 mM phosphate buffer buffer (pH 7.0) was used for recording the spectra. Protein concentration was 3.0 µM for the experiment and measurement was carried out at 25°C. For monitoring tyrosine fluorescence the excitation wavelength of 278 nm was used and the spectra were recorded between 300-400 nm