Chapter 3

Materials & Methods
3.1. Materials

3.1.1. Bacterial strains, plasmids and markers

All the bacterial strains used in the present study are listed in Table 3.1 and 3.2 along with their relevant markers, source, and reference. Table 3.3 and 3.4 represent the plasmids and molecular weight markers used in the study.

Table 3.1 Mycobacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium tuberculosis</em> H37Rv</td>
<td>TMC 102</td>
<td>CDRI, Lucknow</td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em> BCG</td>
<td>Pasteur Strain (Isolate1173P2)</td>
<td>Institute Pasteur, Lille</td>
</tr>
<tr>
<td><em>Mycobacterium fortuitum</em></td>
<td>ATCC 6841</td>
<td>Central JALMA Institute for Leprosy, Agra, India</td>
</tr>
</tbody>
</table>

Table 3.2 *Escherichia coli* Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F′Φ80d lac ZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsd R17 (rKm+) phoA supE44 lacZ15 T7 promoter expression host</td>
<td>Transformation and growth of plasmids.</td>
<td>BRL</td>
</tr>
<tr>
<td>NM522</td>
<td>F′proA Δ lacI9 Δ(lacZ)M15 Δ(lac-proAB)glnV thi-1 Δ(hsdS-mcrB)5</td>
<td>Transformation and growth of plasmids</td>
<td>NEB</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F′ompT hsd S B (rB′ mB′) gal dcm (DE3)</td>
<td>T7 promoter expression host</td>
<td>Novagen</td>
</tr>
</tbody>
</table>
Materials and Methods

| BL21(DE3) pLysS | F'ompT hsd S\textsubscript{B} (r\textsubscript{B}, m\textsubscript{B})
gal dcm (DE3) pLysS (Cm\textsuperscript{R}) | High stringency T7 promoter expression host | Novagen |
| Tuner\textsuperscript{TM} (DE3) | F'ompT hsd S\textsubscript{B} (r\textsubscript{B}, m\textsubscript{B})
gal dcm lacY1 (DE3) | High stringency T7 promoter expression host
uniform dose dependent IPTG induction | Novagen |
| TOP10 | F'mcrA Δ(mrr-hsd RM5 mcrBC) Φ80 lacZΔM15
ΔlacX74 deoR recA1 araD
Δ(ara-leu) 7697 galU gal K
rpsL (str\textsuperscript{R}) endA1 nupG | Host strain for TOPO-cloning vectors | Invitrogen |

Table 3.3 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Phenotype/character</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18, pUC19</td>
<td>Amp\textsuperscript{r}</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>pMV261</td>
<td>Km\textsuperscript{r}, OriM, P\textsubscript{hsp60}</td>
<td>Stover \textit{et. al.} (1991)</td>
</tr>
<tr>
<td>pMV306</td>
<td>Km\textsuperscript{r}, att, int</td>
<td>Stover \textit{et. al.} (1991)</td>
</tr>
<tr>
<td>pCDlux</td>
<td>km\textsuperscript{r}, att, int, P\textsubscript{hsp60}-lux</td>
<td>Deb \textit{et al.}, 2000</td>
</tr>
<tr>
<td>pLL192</td>
<td>Str\textsuperscript{r}, gfp-kan, OriE, OriM</td>
<td>Srivastava \textit{et al.}, 2007</td>
</tr>
<tr>
<td>pMV261</td>
<td>km\textsuperscript{r}, OriM, P\textsubscript{hsp60}</td>
<td>Stover \textit{et. al.}, 1991</td>
</tr>
<tr>
<td>pMV361</td>
<td>km\textsuperscript{r}, OriM, P\textsubscript{hsp60}</td>
<td>Stover \textit{et. al.}, 1991</td>
</tr>
<tr>
<td>pTOPO 2.1</td>
<td>Amp\textsuperscript{r}</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pET19b</td>
<td>Amp\textsuperscript{r}</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pLL192::Phsp60</td>
<td>Str\textsuperscript{r}, P\textsubscript{hsp60}-gfp- kan, OriE, OriM</td>
<td>Srivastava \textit{et al.}, 2007</td>
</tr>
</tbody>
</table>
Table 3.4 DNA Markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fragment size bp</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ DNA/ HindIII Digest</td>
<td>23130, 9416, 6557, 4361, 2322, 2027, 564, 195</td>
<td>MBI, Fermentas</td>
</tr>
<tr>
<td>λ DNA/ HindIII EcoRI Double Digest</td>
<td>21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125</td>
<td>MBI, Fermentas</td>
</tr>
<tr>
<td>50bp Ladder</td>
<td>1000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50</td>
<td>MBI, Fermentas</td>
</tr>
<tr>
<td>100bp Ladder Plus</td>
<td>3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100</td>
<td>MBI, Fermentas</td>
</tr>
<tr>
<td>1kb Ladder</td>
<td>10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250</td>
<td>MBI, Fermentas</td>
</tr>
</tbody>
</table>

Table 3.5 SDS-PAGE Protein markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fragment size kDa</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Ladder (SM0671)</td>
<td>180, 130, 100, 73, 54, 50, 24, 16, 10</td>
<td>MBI, Fermentas</td>
</tr>
</tbody>
</table>

3.1.2. Culture Media and Buffers

The composition of buffers, antibiotics, dyes, and stains used in the present study are listed in the appendix.
3.1.3. Chemicals and Biochemicals

Analytical and molecular biology grade chemicals and general molecular biology products including antibiotics and enzymes, specific enzyme tagged antibodies, proteinase K and protein molecular weight markers were purchased from Sigma, USA. The restriction endonucleases and other DNA enzymes were purchased from MBI Fermentas and New England Biolabs. Custom oligonucleotides were synthesized from Sigma Genosys. PCR and RT-PCR kits were procured from Qiagen.

Cy2, Cy3 and Cy5 were from GE Healthcare, USA; pharmalytes (pH 3-10, pH 4–7), dry strips (pH 4–7) and cover fluid were from GE Healthcare, USA; DMF (Spectrochem, Mumbai India); SDS, CHAPS, Urea, Tris, Acrylamide, Bis- acrylamide, TEMED, DTT and lodoacetamide, bromophenol blue (Amresco); FA (Central Drug House, New Delhi); Trypsin (sequencing grade) was obtained from GE Healthcare, USA; ACN and Water (HPLC grade) was purchased from G Biosciences, USA.

Dehydrated media and ingredients from HiMedia and Difco, and general chemicals and solvents for routine use were used from Merck (Germany), BDH (England), BRL (USA), and local companies like Qualigens, Spectrochem, HiMedia and Ranbaxy.

3.1.4. Water

Milli RO (Reverse Osmosis) grade water (Millipore) was used to get high quality water. Milli RO grade water 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/RNA analysis.

3.1.5. Primers

I. Primers for cloning of rpf genes in pET19b

a) Primers for cloning of rpfC
   
   RpFC F: 5’-TCATATGGGTCCCAGCCCGAACTGG-3’
   RpFC R: 5’-CATGGGATCCGTCAGCGGAATACTTG-3’

b) Primers for cloning of rpfE
   
   RpFE F: 5’-TGATATGGACGACGCGGTCTTGA-3’
   RpFE R: 5’-TCTTTGATCCTATCAGCCCGCGCGCGCGCA-3’
II. Primers for cloning of rpf genes under Phsp60 in pMV361

a) Primers for cloning of rpfC
   3RpfC F: 5'-CGGAATTCCATCCTTTGCCGGCGGCAG-3'
   3RpfC R: 5'-CGGAATTCTCAGCGCGGAATACTACTTTCGC-3'

b) Primers for cloning of rpfE
   3RpfE F: 5'-CGGAATTCAAGAACGCCCGGTACGACGCTC-3'
   3RpfE R: 5'-TCAGCCGCAGGCGGCCGCA-3'

III. Primers for cloning 5' upstream region of rpf genes in pLL192

a) Primers for cloning of rpfC gene
   RC F: 5'-CGGGATCCTCGCGATGGGTGACGCC-3'
   RC R: 5'-CGGGATCCGTCGGCCGGCAAAGGATGCAC-3'

b) Primers for cloning of rpfE gene
   RE F: 5'-GAAGATCTCGCGTCGACACGTTGGTGCGT-3'
   RE R: 5'-GAAGATCTCGTCGACGGGCGTTCTTCAA-3'

IV. Primers used for expression profiling of rpf genes by Real time RT-PCR in stress conditions

PRC F: 5'-ATGAACGAGGCGCTCGA-3'
PRC R: 5'-GGTATATGCTGCCTATCG-3'
PRE F: 5'-GATCGCTAAGAGATCCGCCG-3'
PRE R: 5'-GCCGAAGAGAAACACAGA-3'

V. M13 Standard Primers

M13F: 5'-GTAAAACGACGGCCAG-3'
M13R: 5'-CAGGAAACAGCTATGAC-3'
16SrRNAF: 5'-TCCCGGGCCTTGTACACA-3'
16SrRNAR: 5'-CCACTGGGCTCGGGTGTGA-3'
PHSP60N: 5'-GAAGATCTGTCGATACGTCGACACGATTAC-3'
PHSP60C: 5'-GAAGATCTGTCGATACGTCGACACGATTAC-3'
pMV261seq1: 5'-TCCGTCGCGCCGACTGAC-3'
pMV261seq2: 5'-ACAACCTTGAGCGCCATGAC-3'
3.2. General Methods

3.2.1. General Methods for Recombinant DNA

3.2.1.1. PCR amplification

The PCR reagents from Invitrogen/Qiagen were used for amplification. A reaction was set up according to the manufacturer instructions and condition as given below. The reaction mixture contained:

- 10X Taq/Pfu DNA polymerase buffer: 5 μl
- dNTP mix: 200 μM each
- Primers: 25 pmoles each
- MgCl₂: 1.5 mM
- Taq/Pfu DNA polymerase: 1.5 units
- Template DNA: 0.10-10 ng
- Milli Q water: to 50 μl (final volume)

The general amplification conditions for \( rpfC \) and \( rpfE \) genes are given below:

- Initial denaturation: 95 °C for 5 min \{ 1 cycle
- Denaturation: 96 °C for 1 min
- Annealing: 52 °C for 1 min \} 30 cycles
- Extension: 72 °C for 1 min
- Final extension: 72 °C for 10 min \} 1 cycle

3.2.1.2. Agarose Gel Electrophoresis

DNA was analyzed using horizontal submarine gel electrophoresis apparatus (BRL or Genei) as described by Sambrook et al. (2001). The gel was prepared by weighing and melting appropriate amount of agarose powder in 1X TAE/TBE buffer and casting it into a tray fitted with a comb. Solidified gel was placed in an electrophoresis tank filled with 1X TAE/TBE buffer and DNA samples were mixed with gel loading dye and loaded into the wells. Electrophoresis was carried out at a constant voltage of 5-10 V/cm. After the
required separation, DNA was stained with ethidium bromide and visualized under UV light. The electrophoresed DNA was analyzed on UVP Gel Documentation system (Bio-Rad) and photographed. In case where the DNA bands need to be eluted, preparative agarose gels were prepared using either routine or low melting agarose and eluted by gel extraction kit (Qiagen) according to manufactures instruction.

3.2.1.3. Digestion of DNA using Restriction Endonucleases

DNA was digested with appropriate restriction enzyme in buffer recommended by the manufacturer. 1 μg of DNA was digested in a total volume of 30 µl for 3 h at temperature optimal for respective enzyme(s). The reaction was terminated by heating at 75°C for 10 min or by the addition of tracking dye and freezing at -20°C. Digestion product was analyzed by agarose gel electrophoresis with DNA molecular size markers.

3.2.1.4. Quantitation and purification of DNA

The quantity of DNA was determined by measuring O.D. at 260 nm (1 O.D. = 50 μg/ml for double stranded DNA, 1 O.D. = 33 μg/ml for single stranded DNA). The DNA was purified either by simple phenol:chloroform:isoamyl alcohol extraction or ethanol precipitation (Sambrook et al., 2001), depending on the need. After purification, DNA was air-dried and suspended in appropriate volume of Milli Q water for further use.

3.2.1.5. Methods for cloning of ORFs in different vectors

3.2.1.5.1. PCR amplification as in section 3.2.1.1.
3.2.1.5.2. Agarose gel electrophoresis as in section 3.2.1.2.
3.2.1.5.3. Digestion of DNA using restriction endonucleases as in section 3.2.1.3.

3.2.1.5.4. Dephosphorylation of vector DNA

The vector used for cloning and in some cases the inserts were digested with appropriate restriction enzyme according to manufacturer’s instructions and then dephosphorylated using calf intestinal alkaline phosphatase (CIAP). Dephosphorylation was carried out in the CIAP buffer (MBI Fermentas). One unit of CIAP enzyme was added for 0.15 pmole ends of vector and the reaction mixture was kept for 45 min at 37°C. The mixture was extracted with phenol and then with chloroform:isoamyl alcohol. DNA in
aqueous phase was precipitated by addition of equal volume of isopropanol in the presence of 0.3 M sodium acetate, pH 5.2, washed twice with 70% ethanol, air-dried and suspended in deionized water.

3.2.1.5.5. Ligation

A. Ligation of staggered ends

Ligation was carried out according to manufacturer’s instructions (MBI, Fermentas). The insert to vector ratio was 3:1 pmole ends in most cases. The reaction mixture of 10-20 μl final volume contained:

- Insert DNA: 3x pmole ends
- Vector DNA: 1x pmole ends
- 10X T<sub>4</sub> DNA Ligase buffer: 2.0 μl
- ATP (10mm): 2.0 μl
- PEG 4000 (50%): 2.0 μl
- T<sub>4</sub> DNA Ligase: 2-5 Units

The reaction was carried out at 16-22°C for 15-18 h followed by incubation at 65°C for 10 min to inactivate ligase before transformation.

B. Ligation of blunt ends

The reaction conditions were the same except that the vector-insert ratio and incubation time were further optimized. The reaction mixture of 10-20 μl final volume contained:

- Insert DNA: 0.2 μg
- Vector DNA: 0.1-0.5 μg
- 10X T<sub>4</sub> DNA Ligase buffer: 2.0 μl
- ATP (10mm): 2.0 μl
- PEG 4000 (50%): 2.0 μl
- T<sub>4</sub> DNA Ligase: 5 Units
The reaction was carried out at 16°C for 15-18 h followed by inactivation of ligase at 65°C for 10 min before transformation.

3.2.1.5.6. Transformation of plasmid in *E. coli*

DH5α and NM strain of *E. coli* were used for maintenance of all the plasmids and recombinant molecules. Transformation was done according to Hanahan (1983) with minor modifications. Briefly, an overnight seed culture of *E. coli* was prepared in LB at 37°C with shaking. The seed culture was diluted 100 fold in 10 ml fresh LB and grown in shaking incubator at 37°C till absorbance at 600 nm (A₆₀₀) of 0.5-0.6. The cells were pelleted by centrifugation at 5000 x g for 10 min at 4°C, washed with 2.5 ml chilled transformation buffer I (TFBI) and finally re-suspended in 2.5 ml transformation buffer II (TFBII). The cells in TFBII were incubated over ice for 45 min to make the bacterial cells competent for transformation. Cells were again centrifuged and pellet was suspended in 0.4 ml fresh TFBII. For transformation, 1 μg DNA was added to the competent cells and incubated over ice for 30 min. Heat shock of 45 sec was given in a water bath at 43.5°C. Immediately after heat shock, the cells were rejuvenated by suspending in Z broth. The transformants were then selected by plating onto selective antibiotic plates.

3.2.1.5.7. Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated by the alkaline lysis method (modified from Sambrook *et al.*, 2001). Late log overnight culture of *E. coli* harbouring the plasmid was harvested by pelleting the cells at 6000 x g for 10 min. The pellet was suspended in GTE buffer (1x). 2x volumes SDS-NaOH was then added to the suspension for lysis of the bacteria for 5 min. The solution was then neutralized with 3 M sodium acetate pH 5.2. Lysate was well mixed by inverting the tube several times gently and incubated on ice for 1 h. The resulting precipitate was then removed by centrifugation at 12000 x g for 20 min. Plasmid DNA from the supernatant was precipitated by adding equal volume of isopropanol and centrifugation at 12000 x g for 30 min. The DNA pellet was washed twice with 70% ethanol and once with 96% ethanol and dried. The dried pellet was suspended in deionized water.
3.2.1.5.8. Nucleotide Sequencing of Cloned Insert

The plasmid or PCR product to be sequenced is used as template for setting reaction mix. This product is isolated or purified using Qiagen kits as per instructions by the manufacturer. The sequencing reaction of 20 µl final volume contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td></td>
</tr>
<tr>
<td>PCR product (10-100 ng)</td>
<td></td>
</tr>
<tr>
<td>Primer</td>
<td>5 pmole</td>
</tr>
<tr>
<td>Big Dye Terminator reaction mix</td>
<td>4 µl</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>to final volume of 20 µl</td>
</tr>
</tbody>
</table>

The reaction mixture was mixed properly and subjected to the following PCR cycle:

- Initial denaturation: 95°C for 4 min
- Denaturation: 94°C for 20 sec
- Annealing: 50°C for 20 sec
- Extension: 60°C for 4 min

30 cycles

The amplified product was precipitated with 0.3 M sodium acetate (pH 4.7-5.2) and 2.5 volumes of filtered 96% ethanol. The DNA was pelleted by centrifugation at 12000 x g for 30 min at room temperature, washed with fresh 70% ethanol and air-dried for 30 min. DNA was resuspended in 20 µl of TSR (Template Suppressor Reagent). The sample was denatured at 94°C for 3 min, kept on ice and transferred to 0.5 ml sequencing sample vials. The sample tubes were placed in the sample tray and loaded onto ABI Prism 310 Genetic Analyser for sequencing.

3.2.1.6. Isolation of genomic DNA from mycobacteria

Mycobacterial cultures were grown in Sautons’s medium or on Lowenstein Jensen (LJ) slants at 37°C till log phase (4-6 months for a slow growing species). The cells were scrapped from LJ or pelleted from liquid media, suspended in Tris-EDTA sodium chloride (TES) and heat inactivated at 80°C for 1 h. The cells were treated with lysozyme (2 mg/ml) at 37°C for 2 h to overnight. After incubation, sodium dodecyl sulphate (SDS,1.5%) and proteinase K (100 µg/ml) were added and kept at 50°C for 1 h. Equal volume of buffered phenol was added, mixed and kept on ice for 10 min. Two phases were
formed which were separated by centrifugation at 12000 x g for 20 min. The aqueous layer was removed and extracted twice with chloroform:isoamyl alcohol. DNA present in the aqueous phase was precipitated by addition of equal volume of isopropanol and pelleted by centrifugation at 12000 x g for 15 min at 4°C. The DNA pellet was washed twice with 70% ethanol and once with 96% ethanol, air-dried and suspended in Tris-EDTA (TE) buffer.

3.2.1.7. Isolation of plasmid DNA from Mycobacteria

Mycobacteria containing the plasmid were inoculated in Sautons’s or MB7H9-ADC broth with an appropriate antibiotic and incubated at 37°C till late log phase. The cells were then harvested by centrifugation (6000 x g for 5 min at 4°C) and the supernatant was discarded. The pellet was resuspended in GTE and lysozyme (2 mg/ml final concentration) and incubated at 37°C for 1 h to overnight. Two volumes of SDS-NaOH mixture were added and the mixture was incubated at 45°C for 5 min and there after on ice for 5 min. 1.5 volumes of 3 M sodium acetate pH 5.2 was added to the mixture and incubated for 1 h on ice. The resulting precipitate was removed by centrifugation at 12000 x g for 20 min. The plasmid in the supernatant was precipitated by adding equal volumes of isopropanol and incubated overnight at -20°C. The plasmid was pelleted by centrifugation, washed twice with 70% ethanol and once with 96% ethanol. The pellet was air-dried and resuspended in deionized water. It was then stored at -20°C.

3.2.1.8. Acid Fast Bacilli (AFB) Staining

A loopful of mycobacterial culture was emulsified in saline water on a clean glass slide, air-dried and heat fixed. The smear was stained using carbol fuchsin and heated for 5-7 min till vapors appeared. The slide was cooled and excess stain was washed with tap water and decolorized 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 while the background and contaminating organisms stain green.
3.2.1.9. Electroporation of plasmid DNA in Mycobacteria

Transformation of plasmid in mycobacterial cells was done by electroporation as described by Pelicic et al. (1997). Mycobacterial cells from seed culture were inoculated in 100 ml Sauton’s or MB7H9-ADC broth and incubated at 37°C till absorbance at 600 nm (A600) reached 0.5-1.0 in stationary or shaking condition (7 to 14 days). The purity of culture was checked by AFB staining. Cells were pelleted at 3000 x g for 10 min at room temperature, supernatant was discarded and the cell pellet was resuspended in chilled 10% glycerol solution. Cells were again pelleted by centrifugation and washed twice with chilled 10% glycerol and finally suspended in 2 ml 10% glycerol. 0.4 ml of this suspension was added to each eppendorf tube containing 500 ng DNA and incubated for 5 min in ice. The solution was transferred into sterile electroporation cuvettes with 0.2 cm electrode gap. The cuvettes were tapped to get the cells between the electrode plates and remove air bubbles. Cuvettes were then placed in the electroporator (Bio-Rad, Gene Pulser) and exposed to a pulse of 2.5 kV, 25 μF and 1000 Ω, after which 2.6 ml of Sauton’s medium was added. Cells were incubated overnight at 37°C and plated on to specific selective media.

3.2.1.10 Rapid analysis of recombinant plasmids in E.coli

This process was developed by Sambrook and his colleagues in 2001. Briefly, in this method, Cells were scrapped from the colony with the help of loop and transferred to an eppendorf tube containing 50 μl of suspension buffer and vortexed. 50 μl of freshly prepared lysis buffer was added, vortexed and incubated at 70°C for 5 min followed by the addition of 1.5 μl of KCl (4 M) and 0.4% bromophenol blue. The mixture was incubated on ice for 5 min, centrifuged at 12000 x g for 3 min at 4°C and 50 μl of the supernatant was loaded on to a 0.7% agarose gel and analyzed for plasmid size. Plasmid without insert processed similarly was used as control.
3.2.2. General methods for Protein Expression, Purification and Characterization

3.2.2.1. PCR amplification of rpfC and rpfE genes

The two rpf genes from \textit{M. tuberculosis} were amplified from H37Rv DNA using the specific primer (Materials and Methods, Section 3.1.5) each gene lacking their 5' ends, which codes for signal sequences. \textit{NdeI} and \textit{BamHI} restriction sites were incorporated in primers for cloning. The amplification condition is as follows.

\begin{verbatim}
Initial denaturation 95°C for 5 min } 1 cycle
Denaturation 94°C for 1 min
Annealing 55°C for 1 min \{ 30 cycles
Extension 72°C for 2 min
Final extension 72°C for 10 min } 1 cycle
\end{verbatim}

The amplified products were of 331 bp (rpfC) and 435 bp (rpfE).

3.2.2.2. Cloning of rpf genes in pET19b

The amplified fragments of rpfC and rpfE were first cloned in pTOPO 2.1 TA cloning vector and sequenced using M13F (Forward) and M13R (Reverse primers). All fragments were excised from the cloning vector by digestion with \textit{NdeI} and \textit{BamHI} and ligated to \textit{NdeI} and \textit{BamHI} cleaved pET19b expression vector. The vector contains T7 promoter, LacI repressor and ampicillin as selection marker.

3.2.2.3. Optimizing expression and solubilization of protein

The conditions for protein induction were optimized before starting protein expression, so as to maximize the partitioning of protein in soluble fraction. Following parameters were taken into consideration.
A. *E. coli* host strains
   BL21 (DE3), BL21 (DE3) pLysS, Tuner (DE3)

B. IPTG concentrations
   0-1000 µM

C. Incubation temperatures after induction
   16-37°C

D. Incubation times after induction
   2-20 h.

3.2.2.3.1. Induction of Proteins

A single bacterial colony was picked up from a freshly streaked plate and inoculated in LB broth containing appropriate antibiotic and grown overnight at 37°C with shaking. The culture was diluted (50 fold) in fresh LB broth (pH 7.2) containing antibiotic and incubated till an $A_{600}$ of 0.5-0.8. The culture was induced by the addition of 0.5 mM IPTG and further incubated at 37°C for 4 h. The cells were harvested by centrifugation at 3000 x g for 10 min at 4°C. The supernatant was discarded and the pellet was suspended in 10 mM Tris-HCl (pH 8.0). The cell pellet was processed for the preparation of total cell protein, soluble and insoluble fractions.

3.2.2.3.2. Sample preparation for protein profile analysis

The induction of the protein and partitioning of recombinant protein into different cellular fractions was analyzed as follows.

A. Total cell protein sample

The induced cell sample prepared as above was mixed with equal volume of 2X Laemmli sample buffer. The mixture was heated for 5 min in boiling water bath and centrifuged at 12000 x g for 5 min. The supernatant was analyzed by SDS-PAGE (Poly Acrylamide Gel Electrophoresis).

B. Soluble cytoplasmic fraction

The induced cell pellet suspension in 10 mM Tris-HCl (pH 8.0) was treated with lysozyme (100 µg/ml) followed by sonication for cell lysis. The disrupted cells were centrifuged at 12000 x g for 10 min. The supernatant contained soluble cytoplasmic fraction while pellet contained insoluble fraction. The supernatant containing the soluble
cytoplasmic fraction was mixed with equal volume of 2X Laemmli sample buffer. The mixture was heated for 5 min in boiling water bath and used for analysis by SDS-PAGE.

C. Insoluble cytoplasmic fraction

The pellet obtained after removal of soluble cytoplasmic fraction was resuspended in 10 mM Tris-HCl (pH 8.0) containing 1% SDS with heating and vigorous mixing. The mixture was mixed with equal volume of 2X Laemmli sample buffer, heated for 5 min in boiling water bath and used for analysis by SDS-PAGE.

3.2.2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The vertical slab gel unit was assembled using the manufacturer's instructions (Bio-Rad). The resolving monomer solution of desired percentage of acrylamide was prepared in Tris-HCl 0.375 M (pH 8.8) containing 0.1% SDS, 0.05% APS and 0.05% TEMED. The gel was casted by pouring the monomer solution into the mould in a steady stream and overlaid using water or water saturated butanol solution. After polymerization at room temperature, monomer solution of acrylamide in 0.125 M Tris, pH 6.8 containing 0.1% SDS, 0.05% APS and 0.05% TEMED was overlaid as 3.5% stacking gel. Appropriate sized gel combs were introduced at the top of the stacking gel and the gel was allowed to polymerize at room temperature.

After polymerization the comb was removed and the sandwich containing the SDS-PAGE was fixed with the buffer unit of the apparatus. The upper and lower chambers of the tank were filled with Tris-Glycine buffer and the samples were loaded after cleaning the well with the tank buffer. The gel was electrophoresed initially at 80 V till the samples reached resolving gel and thereafter at 120 V till the end.

The gel was removed from the apparatus and stained using coomassie brilliant blue R-250 solution for 2 h at room temperature and destained with gentle shaking. The gel bands were analyzed along with appropriate markers and documented by gel documentation system (UVP) and stored for further reference in a gel storage solution.

3.2.2.5. Western blot analysis

The western blots were prepared by transferring the separated proteins from the polyacrylamide gel onto a nitrocellulose membrane in a Trans blot apparatus (Bio-Rad Mini protean III) as described by Towbin et al. (1979). After electrophoresis the gel was soaked in transfer buffer for 10 min and then sandwiched between Whatman paper No.3
on one side and nitrocellulose membrane (Sigma) on other side. Additional sheets of Whatman paper No.3 were put on both sides, fixed tightly in Trans-blot apparatus. Care was taken to avoid any trapped air bubbles between the gel and the nitrocellulose membrane. The apparatus was placed in such a way that the nitrocellulose membrane faces the anode. The transfer was carried out at 50 V for 4 h in cold. Complete transfer of proteins onto the membrane was visually checked by transfer of the colored marker or by Ponceau stain. The membrane was incubated in blocking buffer for 3-4 h followed by incubation with Anti-His antibody conjugate solution (dilutions according to manufacturer's instructions, Clonetech) for 1 h at room temperature with gentle shaking. The blot was washed three times with buffer, for 15 min each. The blot was finally rinsed and soaked in developing solution in dark. After development the blots were rinsed with distilled water, dried and stored.

3.2.2.6. Large-scale purification of recombinant protein using His Bind Resin

All the steps of purification were carried out at 4°C. Induced cultures were harvested by centrifugation and the pellet was suspended in binding buffer (20 mM Tris-HCl (pH 8.0), 10 mM imidazole (pH 7.9), 500 mM NaCl) containing 1 mM PMSF (Boehringer Mannheim) and 100 μg/ml of lysozyme (Sigma). The cells were disrupted by sonication and cell debris was removed by centrifugation at 15,000 x g for 30 min. A 5 ml column was packed with Ni++-NTA resin (Qiagen) and equilibrated with 2 volume of binding buffer. The supernatant containing soluble protein was loaded onto the column. The column was washed with 10 volume of binding buffer and 15 volume of wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 60 mM imidazole). The protein was eluted in the buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 500 mM imidazole. The eluted fraction containing purified protein was dialysed against 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl for activity assays.

The proteins which were expressed as insoluble fraction were purified using urea. 6 M urea was used in all the three; binding buffer (20 mM Tris-HCl (pH 8.0), 10 mM imidazole (pH 7.9), 500 mM NaCl), wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 60 mM imidazole) and elution buffer containing Tris-HCl (pH 8.0), 200 mM NaCl and 500 mM imidazole (pH 7.9).
3.2.3. General methods used for Animal studies / in vivo studies

3.2.3.1. Animals

Female BALB/c mice (18-20 g in weight) were used. Mice were kept in a pathogen-free sterile environment maintained in Institute's animal house facility. All animal protocols employed were approved by the Institutional Animal Ethics Committee (IAEC).

3.2.3.2. Inoculum preparation

Inocula for infection were prepared from the mid-exponential-growth phase cultures of *M. fortuitum* and Recombinant *M. fortuitum* grown under standard laboratory conditions in MB7H9 broth. When required media were supplemented with 25 μg/ml of kanamycin. The cells were grown to mid logarithmic phase, pelleted by centrifugation, washed and resuspended in Normal-saline containing 0.05% Tween 80 (TNS). Mice were inoculated intravenously via lateral tail vein with a standardized inoculum dosage of $5 \times 10^7$ CFU/mice (Parti *et al.*, 2005). 24 h after infection, three mice from each group were sacrificed to determine bacillary load (day 1 CFU count). Animals were monitored daily for appearance or disappearance of visible symptoms of infection and mortality, until 60 days post-infection (PI). The day of infection was taken as day 0.

3.2.3.3. Determination of bacillary load / CFU count

To determine the bacillary load or number of colony forming units per gram kidney tissue (CFU/g tissue) at different time points of infection, a minimum of six mice were sacrificed at each time point. Kidney from infected mice were removed aseptically and homogenized in 2 ml of TNS using glass homogenizer. Tissue bacillary load was quantitated by plating serial dilutions of the kidney homogenates on MB7H10 agar (Difco) or NAT (Nutrient agar (Difco) containing 0.05% Tween 80). Each experiment was repeated at least three times.

3.2.3.4. Histopathological study of kidney tissues

For histopathological comparison and analysis of tissue sections, mice were sacrificed and kidneys removed aseptically, and stored in 10% formalin prepared in
Normal-saline. Representative pieces of kidney tissues were processed for paraffin embedment, sectioning and hematoxylin and eosin staining for histopathology as described previously (Job and Chacko, 1986). Briefly, the tissues were fixed in 10% neutral-buffered formalin, dehydrated in graded concentrations of alcohol, cleared in xylene and embedded in paraffin wax. 5 μm thin sections of paraffin-embedded tissues were prepared and stained with haematoxylin and eosin stains or modified Ziehl-Neelson’s methods.

3.3. Standardization of methods for role of rpf genes in \textit{in vitro} resuscitation of dormant mycobacteria

3.3.1. Standardization of an \textit{in vitro} model of Extended Stationary Phase of non-culturable state

3.3.1.1. Construction of recombinant \textit{M. bovis} BCG expressing firefly luciferase

An integrative \textit{E. coli–Mycobacterium} shuttle vector pCDlux (6.05kb), a derivative of pMV361, expressing firefly luciferase (\textit{lux} gene) was used for the study. It contains \textit{aph} gene conferring kanamycin resistance, an \textit{E. coli} origin of replication \textit{OriE}, \textit{attP}, \textit{int} (for integration in mycobacterial genome) gene and a \textit{lux} gene cloned under the control of \textit{Phsp60} promoter (Deb \textit{et al.}, 2000). It was electroporated in \textit{M. bovis} BCG and selection for recombinant clones was made on MB7H10 plates containing kanamycin (15 μg/ml) and OADC. The kanamycin resistant clones were checked for luciferase activity. For measurement of luminescence, 100 μl of culture was mixed with 250 μl of sodium citrate buffer (0.1 M, pH 5.0) and 100 μl of 1 mM luciferin substrate (Promega USA) and measured in Berthold Auto lumat LB953 tube luminometer as relative light units (RLU) for 10 sec (Deb \textit{et al.}, 2000). The map of pCDlux is shown in Figure 3.1.
3.3.1.2. Standardization of \textit{in vitro} conditions of non-culturable, Extended Stationary Phase (ESP) state

For the establishment of extended stationary phase state in recombinant \textit{M. bovis} BCG (\textit{M. bovis} BCG containing integrative vector pCD lux expressing firefly luciferase with \textit{hsp60} promoter), culture was grown in Sauton’s medium and precultures were diluted to an O.D. $A_{600}$ of 0.05 and kept at 37°C without shaking. Growth of the culture was monitored regularly by measuring $A_{600}$, CFU and RLU. CFU was determined by plating serial dilutions on MB7H10 plates in triplicate and RLU was measured as described above. The cells after 5 months of incubation became non-culturable and failed to grow on agar based plate ($A_{600}$, 4.0). The RLU of these cells is very low or negligible (approximately 70-80).
3.3.2. Study of \textit{in vitro} role of \textit{rpf} genes in resuscitation

To evaluate the role of \textit{rpf} genes \textit{in vitro}, an Extended Stationary Phase (ESP) of \textit{M. bovis} BCG was used. The cells in this stage are metabolically inactive, represented by low RLU and also non-culturable on agar based plate. Development of \textit{M. bovis} BCG of this stage involves 5 months of prolonged incubation without shaking. A resuscitation assay was developed using recombinant protein of \textit{rpE} and supernatant of \textit{M. luteus}. Recombinant protein of 100 pmole and supernatant of \textit{M. luteus} were used to resuscitate the non-culturable cells of BCG.

3.3.2.1. Preparation of supernatant of \textit{M. luteus}

Supernatant of \textit{M. luteus} was used to reactivate the ESP cells of \textit{M. bovis} BCG. \textit{M. luteus} cells were first grown in LBGT (Luria Bertani broth containing 0.05% Tween 80 and 0.2% glycerol) for inoculum preparation. The preculture was diluted to $A_{600}$ 0.05 in LMM (Lactate Minimal Medium). Cells were grown with shaking at 250 rpm till $A_{600}$ of this culture reached 1.8-2.0. The cells, at this stage were removed by centrifugation at 8000 x g for 30 min at 4°C and supernatant (SN) was filter sterilized through 0.2 μm filter and used within 2 h of preparation as an endogenous source of Rpf protein (Cohen-Gonsaud \textit{et al.}, 2005). The filtered supernatant was used for resuscitation assay.

3.3.2.2. Preparation of recombinant protein RpE

Recombinant protein RpE was purified by affinity chromatography using His tag column as described earlier. 100 pM of this protein was used in the resuscitation assay.

3.3.2.3. Standardization of Resuscitation assay

Resuscitation assay was standardized in 24 well plates (NUNC). Recombinant protein RpE and supernatant were made first. Non-culturable cells containing only Sautons’s medium and LMM served as control. In first lane, 50 μl non-culturable cells of \textit{M. bovis} BCG was incubated with the 200 μl of Sautons’s medium and 200 μl of LMM. Second lane contained, 50 μl non-culturable cells of \textit{M. bovis} BCG, 200 μl of Sautons’s medium and 200 μl supernatant of \textit{M. luteus} containing endogenous Rpf protein. In the third lane 100 pmoles of recombinant protein RpE were added to 400 μl of Sauton’s medium containing 50 μl of non-culturable. The plates were kept at 37°C without shaking.
To measure the resuscitation of non-culturable cells, $A_{600}$ and RLU were measured at interval of 12, 24, 48, 192, 264, and 432 h.

The resuscitation assay was also performed on large scale for isolation of RNA and proteome analysis by 2-D gel electrophoresis using DIGE. The assay was developed in 500 ml flask. To make RSP cells, 5ml ESP cells were added to 100 ml of *M. luteus* supernatant (SN) and 100 ml of Sauton's media. The cells ($A_{600}$, 0.225) were incubated at 37°C without shaking. The control ESP cells contained LMM media instead of SN. Cells were monitored for Resuscitation by measuring $A_{600}$ and RLU for 25 days at regular intervals. Cells grown with SN were labeled as RSP and those without SN were represented as ESP cells.

### 3.4. Methods for establishing of role of *rpfC* and *rpfE* genes in *in vivo* model of Persistent infection

To investigate the *in vivo* role of *rpf* genes, *rpfC* and *rpfE* genes were cloned under the control of constitutively expressed promoter *hsp60* in pMV361 vector and the constructs were used to infect mice in a murine infection model of persistence developed in our lab (Parti *et al.*, 2005).

#### 3.4.1. Development of *M. fortuitum* murine infection model

(Adapted and modified from Parti *et al.*, 2005)

Female BALB/c mice were injected intravenously with a standardized dosage inoculum of $5 \times 10^7$ CFU of *M. fortuitum*. Mice were monitored regularly for appearance of the disease symptoms and mortality. The tissue bacillary load in kidney was determined at different time points (5, 10, 25, 40, and 60 days post-inoculation). A reliable and reproducible murine infection model was developed in which non-replicating persistence of $10^5$ CFU/g tissues in kidney was observed. Histopathology of the kidney tissue at different time points was done as described.
3.4.2. Construction of recombinant *M. fortuitum* expressing *rpfC* and *rpfE* genes

3.4.2.1. Amplification of *rpfC* and *rpfE* genes

The *rpfC* and *rpfE* genes without stop codon were amplified from the genomic DNA of *M. tuberculosis* H37Rv. The primers (Materials and Methods, Section 3.1.5.) and the conditions used for amplification are as follows:

Initial PCR Activation step \[95^\circ C \text{ 15 min}\] 1 cycle
Denaturation \[95^\circ C \text{ 1 min}\]
Annealing \[53^\circ C \text{ 1 min}\] 30 cycles
Extension \[72^\circ C \text{ 1 min}\]
Final Extension \[72^\circ C \text{ 10 min}\] 1 cycle

The amplified products of *rpfC* and *rpfE* were of 531 bp and 519 bp respectively.

3.4.2.2. Cloning of *rpfC* and *rpfE* genes in pMV361

pMV361 is an integrative *E. coli* – *Mycobacterium* shuttle vector containing an *E. coli* origin of replication (*OriE*), *ahp* gene conferring kanamycin resistance, *Phsp60*, *attP*, and *int* gene. The PCR amplified fragments of *rpfC* and *rpfE* genes were first cloned into pTOPO 2.1 vector and sequenced using M13F and M13R primers. All fragments were excised from the vector by digestion with *EcoRI* and then cloned into *EcoRI* cleaved CIAP treated pMV361 vector. Right oriented clone of *rpfC* was selected by restriction digestion with *PvuII*. Similarly, right oriented clone of *rpfE* was selected by restriction digestion with *NotI*. The right oriented clone and vector were electroporated into *M. fortuitum* and the selection for recombinant clones was made on NAT plates containing kanamycin (25 µg/ml). The kanamycin resistant colonies were checked by colony PCR. Recombinant *M. fortuitum* possessing vector alone was used as control.

3.4.3. Validation of expression of *rpf* genes in recombinant *M. fortuitum*

3.4.3.1. RNA isolation from recombinant *M. fortuitum* and quantitation by RT-PCR

Total RNA was isolated from recombinant *M. fortuitum* using RNeasy Qiagen kit (Qiagen, Germany). Late log phase culture of all the three recombinant *M. fortuitum*
grown in MB7H9 was taken for isolation of RNA. The cultures were grown till absorbance at 600 nm A$_{600}$ reached 0.6-0.8 and CFU was determined by nephelometer (10$^8$ cells) (Becton Dickinson). RNA was isolated from RNeasy kit as per instruction of the manufacturer. Briefly, bacterial cells were harvested by centrifugation at 6000 x g at 4°C for 15 min, and resuspended in 300 µl of TE containing 3 mg/ml of lysozyme. The mixture was incubated at room temperature for 5 min, and 1050 µl of RLT buffer containing 0.1% β-mercaptoethanol was added. The suspension was vortexed properly and sonicated for 45 sec at 50 W using an ultrathin probe (Heat systems ultrasonics). The tubes were centrifuged for 1 min at 8000 x g at 4°C and the supernatant was transferred to micro centrifuge tubes. 0.7 volume of ethanol was added and after mixing, the solution was applied to RNeasy column. After centrifugation for 15 s at 8000 x g at 20°C, the columns were washed once with 700 µl buffer RW1 and twice with 500 µl of buffer RPE. Subsequently, the column were dried by centrifugation at 8000 x g at 20°C for 2 min and the RNA eluted with 50 µl of RNA free water, RNA analysis was done by formaldehyde-agarose gel electrophoresis.

3.4.3.2. Relative quantification of RNA by semi-quantitative RT-PCR

RT-PCR analyses were carried out using one step RT-PCR kit (Qiagen). The concentration and purity of RNA isolated was determined by measuring absorbance at 260 and 280 nm. RT-PCR without prior reverse transcription was performed to ensure exclusion of DNA contaminants. The specific primers for rpf genes were used for analysis.

For quantitative analysis, samples were taken at three cycle intervals between cycles 18-30 to compare non-saturated PCR product formation. Data were verified in three independent experiments. 16S rRNA was used as internal control. RT-PCR analyses were carried out with the one step RT-PCR kit (Qiagen) as described. The primers and conditions for PCR amplification used for analysis is as follows:

RT-PCR Conditions

A. One-step RT-PCR

Protocol optimized for 200 ng-2 µg total RNA
5x one step RT buffer  
\[10 \mu l\]
dNTP mix (10mm)  
\[2 \mu l\]
Primer 1  
\[1 \mu l\] (0.6 \mu M)
Primer 2  
\[1 \mu l\] (0.6 \mu M)
One-step enzyme mix  
\[2 \mu l\]
RNase inhibitor  
\[5-10 U\]
Template total RNA  
\[2 \mu l\] (500 ng)
RNase free water  
upto 50 \mu l
Reverse transcription  
\[50^\circ C\] 30 min

B. PCR conditions

Initial PCR Activation step  \(95^\circ C\) 15 min \{ 1 cycle
Denaturation  \(95^\circ C\) 1 min
Annealing  \(55^\circ C\) 1 min \}\ 30 cycles
Extension  \(72^\circ C\) 1 min
Final Extension  \(72^\circ C\) 10 min \{ 1 cycle

3.4.4. Infection of mice with recombinant *M. fortuitum*

Inoculum for all the three, *M. fortuitum[pMV361]*, *M. fortuitum[pMV361::rpfC]* and *M. fortuitum[pMV361::rpfE]* were prepared as described. *M. fortuitum[pMV361]* was used as vector control. Briefly, the cells were grown to mid logarithmic phase, pelleted by centrifugation, washed and resuspended in Tween -saline (TNS) buffer containing 0.05% Tween 80. BALB/c female mice (18-20 g in weight) were infected by the intravenous (i.v.) route with \(5 \times 10^7\) CFU. Mice were observed for mortality and disease symptoms which appeared within 8-10 days post- infection (PI). The symptoms included spinning of head, twitching, tilting and restlessness. At different time points, 3 mice were sacrificed per group and kidneys were removed aseptically. The kidneys were homogenized in 2 ml of TNS and the homogenate was used for measurement of tissue bacillary load. Tissue bacillary load was determined by plating serial dilutions of kidney homogenate on NAT plates (Parti et al., 2005).
3.4.5. Drug susceptibility of recombinant *M. fortuitum* at different stages of infection

3.4.5.1. Ciprofloxacin sensitivity of bacilli in kidney homogenate

Ciprofloxacin sensitivity of all the three recombinant strains were measured by plating serial dilutions of kidney homogenates on NAT (Nutrient agar supplemented with 0.05% Tween 80) plates containing ciprofloxacin (0.125 µg/ml). NAT plate without ciprofloxacin served as control. Briefly, BALB/c mice were infected intravenously (i.v.) with 5 x 10^7 CFU of *M. fortuitum*[pMV361], *M. fortuitum* [pMV361::rpJC] and *M. fortuitum* [pMV361::rpJE] as described above. Ciprofloxacin (50 mg/kg) in distilled water (0.2 ml) was given by gavage (Oesophageal incubation) daily for 20 days. Controls (Untreated) in each case were dosed with similar volume of normal saline. Three characteristic time points were chosen as starting points for ciprofloxacin treatment during the course of infection for drug susceptibility studies:

(a). Regimen 1: Drug susceptibility of proliferating bacteria-1 day PI

(b). Regimen 2: Drug susceptibility of mid-phase bacteria-25 day PI

(c). Regimen 3: Drug susceptibility of persistent bacteria-40 day PI

At regular intervals, at least six mice were sacrificed from the treated and untreated control groups and kidney homogenates were plated on NAT plates for CFU counts.

3.4.6. Histopathological studies

All the histopathological studies were done as described earlier in general methods. Briefly, the tissues were fixed in 10% neutral-buffered formalin, dehydrated in graded concentrations of alcohol, cleared in xylene and embedded in paraffin wax. 5 µm thin sections of paraffin-embedded tissues were prepared and stained with haematoxylin and eosin stains.

3.4.7. Statistics

Results were presented as mean ±SE. All experiments were conducted at least three times. Statistical comparison for data were made by Student’s t-test and significance inferred at P<0.05.
3.5. Methods for analysis and identification of differentially expressed proteins of *M. bovis* BCG during Extended Stationary Phase (ESP) and Resuscitated phase (RSP)

3.5.1. SDS-PAGE analysis

The buffers and solutions used are listed in appendix.

3.5.1.1. Sample preparation for protein analysis by SDS-PAGE

*M. bovis* BCG cultures grown in Sauton’s medium of Extended Stationary Phase (ESP) and Resuscitated Phase (RSP) were harvested by centrifugation at 6000 x g for 10 min at 4°C, washed twice and suspended in Tris-HCl buffer (10mM, pH 8.0). Cells were sonicated (Heat systems ultrasonics) four times for 30 sec intermittently in ice. Lysed mixture was centrifuged at 10000 x g for 30 min at 4°C and cell debris and membrane were removed and the supernatant containing total soluble protein fraction was procured. The total soluble protein fraction was mixed with equal volume of 2X Laemmli sample buffer, boiled for 5 min in boiling water bath and used for analysis on SDS-PAGE.

3.5.2. 2-Dimensional gel electrophoresis

3.5.2.1. Sample preparation for Two-dimensional (2-D gel) electrophoresis

*M. bovis* BCG cultures of extended stationary phase (ESP) and resuscitated cells (RSP) were harvested by centrifugation at 6000 x g for 10 min at 4°C. Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS), 50 mM dithiothreitol (DTT). The cells were disrupted by sonication in the presence of protease inhibitor cocktail (Roche, Louis, U.K.) and 10 mM PMSF four times for 30 sec intermittently while keeping the tubes in ice. The cell lysate was centrifuged at 10000 x g at 4°C for 30 min and the supernatant was kept as crude protein sample. The crude protein was subjected to precipitation by chilled 80% acetone. The crude protein sample was mixed with four volumes of chilled acetone and kept for 2 h
at -20°C. The protein sample was procured by centrifugation at 12000 x g for 10 min at 4°C, supernatant was discarded and sample was air-dried and finally suspended in lysis buffer and stored in small aliquots at -70°C until they were required.

3.5.2.2. Quantitative measurement of proteins

Lowry's method was used for the estimation of quantity of proteins (Lowry et al., 1951) using Folin phenol reagent. Samples were taken and volume rose to 1 ml with distilled water. 1.0 ml of solution C was added to each tube, vortexed and incubated at room temperature for 15 min. To this was added 100 μl of solution D, vortexed, and incubated at room temperature for 45 min in dark. Absorbance of blue coloured complex formed was measured at 650 nm in spectrophotometer (LKB). Amount of protein present in the test sample was determined from the linear curve obtained for known concentrations of the standard protein (BSA) processed in parallel with the test samples.

2-D protein sample was quantitated by copper iron assay using 2-D Quant kit (GE Healthcare, USA) as per manufacturer’s instruction.

3.5.2.3. Fluorescence labeling of proteins with Cy Dyes for DIGE (Differential Gel Electrophoresis)

After precipitation and estimation, 50 μg of proteins from both ESP and RSP were labeled with 400 pmole of Cy3 or Cy5. Minimal labeling was performed by adjusting the pH of the protein solution to 8.8 with 50 mM Tris-HCl and incubating the protein solution with 400 pmol of Cy dye on ice in dark for 30 minutes. Quenching of the unbound dye was carried out by treating with 1 μl of 10 mM Lysine solutions for 10 min on ice in dark. Dye swapping experiment was carried out so that Cy3 and Cy5 dyes were distributed equally among both ESP and RSP groups. Cy2 was used to label the same amount of internal standard (50 μg), resulting from the pooling of equal amounts of aliquots of both ESP and RSP containing total six samples. Experimental design of labeled proteins with Cy dyes for labeling scheme is shown in the Table 3.6.
Table 3.6 Cy Dye labeling scheme

<table>
<thead>
<tr>
<th>S. No</th>
<th>Cy2</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel 1</td>
<td>Internal standard</td>
<td>ESP 1</td>
<td>RSP 1</td>
</tr>
<tr>
<td>Gel 2</td>
<td>Internal standard</td>
<td>ESP 2</td>
<td>RSP 2</td>
</tr>
<tr>
<td>Gel 3</td>
<td>Internal standard</td>
<td>ESP 3</td>
<td>RSP 3</td>
</tr>
<tr>
<td>Gel 4</td>
<td>Internal standard</td>
<td>RSP 1</td>
<td>ESP 1</td>
</tr>
<tr>
<td>Gel 5</td>
<td>Internal standard</td>
<td>RSP 2</td>
<td>ESP 2</td>
</tr>
<tr>
<td>Gel 6</td>
<td>Internal standard</td>
<td>RSP 3</td>
<td>ESP 3</td>
</tr>
</tbody>
</table>

ESP and RSP 1-3 represent three samples each of ESP and RSP cells of *M. bovis* BCG resuscitated by endogenous Rpf present in the supernatant of *M. luteus*. Internal standard comprise of equal amount of pooled sample of ESP and RSP and labeled with Cy2.

### 3.5.2.4. Sample rehydration

Prior to IEF, labeled samples to be separated on the same gel were mixed and added to rehydrating buffer containing 8 M urea, 2 % CHAPS, 0.002% Bromophenol blue to a final volume of 250 μl. DTT and IPG buffer (pH 4-7) were added to the final concentration of 0.003% and 0.5% respectively. After mixing, samples were centrifuged at 10,000 x g for 2 min to remove any particulate matter. The solution was then loaded on a reswelling tray (GE Healthcare, USA). Immobiline Dry gel strip of pH range 4-7, 13 cm was used for IEF. Plastic cover on the strip was carefully removed and the gel surface was placed over the sample in the tray with forceps. The strips were then overlaid with IEF cover fluid (GE Healthcare, USA) and left 14-16 h at RT in dark for optimum rehydration.

### 3.5.2.5. First dimension electrophoresis (Iso-electric focusing)

Rehydrated IPG strip was kept in a strip holder, overlaid with IEF cover fluid and subjected to iso-electric focusing (IEF) in an Etan IPGphor 3 IEF system (GE Healthcare, USA) as per the following program Table 3.7.
Table 3.7 IEF program

<table>
<thead>
<tr>
<th>STEPS</th>
<th>VOLTAGE MODE</th>
<th>VOLTAGE (V)</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Step</td>
<td>150</td>
<td>1 h</td>
</tr>
<tr>
<td>2</td>
<td>Step</td>
<td>500</td>
<td>30 min</td>
</tr>
<tr>
<td>3</td>
<td>Step</td>
<td>1000</td>
<td>30 min</td>
</tr>
<tr>
<td>4</td>
<td>Gradient</td>
<td>8000</td>
<td>1 h 30 min</td>
</tr>
<tr>
<td>5</td>
<td>Step</td>
<td>8000</td>
<td>6 h</td>
</tr>
</tbody>
</table>

IEF was stopped when a total volt-hours of 30,000 VhT was achieved. Temperature was set at 20°C. Strips were covered with cover fluid throughout the run period.

3.5.2.6. Second dimension separation (SDS-PAGE)

Prior to SDS-PAGE, each strip was equilibrated with SDS equilibration buffer. The Dry strips were incubated in equilibration buffer I containing 6 M Urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8, 1% DTT, 0.002% bromophenol blue for 15 min, followed by equilibration buffer II comprising 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8, 2.5% iodoacetamide, 0.002% bromophenol blue for 15 min. Subsequently, the equilibrated strips were loaded onto a 10% polyacrylamide gel cast on SE 600 Ruby gel apparatus (GE Healthcare, USA). The strips were embedded in 0.5% (w/v) agarose containing bromophenol blue on top of acrylamide gel and run at 20°C, 15 mA per gel for 30 min followed by 30 mA per gel until the bromophenol blue dye front ran off the gel.

3.5.2.7. Image acquisition

After the SDS-PAGE, labeled proteins were visualized using a Typhoon TRIO Variable Mode Imager (GE Healthcare, USA). Cy2 images were scanned using 488 nm excitation and 520BP40 emission filter; Cy3 images were scanned using 532 nm excitation and 580BP30 emission filter; Cy5 images using 633 nm excitation and 670BP30 emission filter. All gels were scanned with a PMT setting of 750 to 800. Images were cropped using Image-Quant™ v 6.5 (GE Healthcare, USA) to remove areas extraneous to the gel image.
3.5.2.8. Software analysis of differentially expressed proteins by DeCyder analysis

3.5.2.8.1. DeCyder analysis

DeCyder software allows the relative quantification of the expression. Expression difference within the gels (ESP and RSP) are first calculated (Differential In-gel Analysis, DIA) and then compared across the gels (Biological Variation Analysis, BVA) using the internal standard (Cy2) image as reference.

3.5.2.8.2. Differential In Gel Analysis (DIA)

Gel images were processed using DeCyder version 6.5. The images were uploaded to the work space using image loader module. These images were imported to DIA workspace to create six different workspaces or each gel pairs for further processing. The maximum number of spots for each co-detection procedure was set to 1500. The spots on gels were co-detected automatically as 2-D DIGE image pairs, which intrinsically link a sample to its in-gel standard.

3.5.2.8.3. Biological Variation Analysis (BVA)

These six DIA workspaces were then imported to BVA workspace for analyzing biological variation. The experimental setup and relationship between samples were assigned in DeCyder software in the BVA workspace. Each individual Cy3 or Cy5 gel image was assigned an experimental condition, either ESP or RSP, and all Cy2 images were classified as standards. The gel with the highest spot count was assigned as the master gel. Matching between gels was performed utilizing the in-gel standard from each image pair. Matching was further improved by land marking and manually confirming potential spots of interest.

3.5.2.8.4. BVA normalization of spot volume and Statistical Analysis

The volume of a spot for a given dye is defined as the fluorescent intensity of the corresponding dye integrated over the area of a spot. Normalized volume refers to the volume normalized across the three dyes and across the gels. One of the outputs provided by DeCyder is the ratio of the normalized volumes, also called the standardized
abundances, \( R_{pg} = \frac{\text{VolCy5}_{pg}}{\text{VolCy2}_{pg}} \), \( G_{pg} = \frac{\text{VolCy3}_{pg}}{\text{VolCy2}_{pg}} \), for each spot \( p \) and gel \( g \) in the experiment. \( \text{VolCy5}_{pg} \) represents the normalized volume of spot \( p \) on gel \( g \) in the Cy5 sample and similarly for the other two dyes. The statistical analyses in DeCyder are based on the standardized protein log abundances, which are defined as the \( \log_{10} \) of the standardized abundances. In theory, the standardized log abundances follow a normal distribution and are comparable across all spots and gels using t-test.

Analysis of protein abundance change between samples was performed using the standardized log abundance values, average ratio of the standardized log abundance was calculated for each spot. The mean of the standardized log abundance of spots on 2-D gels in the case and control gel sets were analyzed using Student’s t-test.

3.5.2.8.5. Average ratio of spots

The average ratio for a given spot between ESP and RSP pools were generated in the DeCyder software. The degree of difference in standardized abundance between two protein spot groups is expressed as average ratio. The average ratio indicates the standardized volume ratio between the two groups. Values are displayed in the range of \(-\infty\) to \(-1\) and \(+1\) to \(+\infty\), values between \(-1\) and \(+1\) are not represented, hence a two fold increase or decrease is represented by 2 and -2, respectively (not 2 and 0.5).

3.5.3. Post electrophoretic staining of gels

3.5.3.1. Silver staining

After SDS-PAGE the gels were immersed in 250 ml fixing solution containing 30 % ethanol, 10 % glacial acetic acid for over night. Once fixation was over, fixing solution was removed and 250 ml sensitizing solution (30 % ethanol, sodium thiosulphate, 5 % (w/v) 10 ml, 6.8 % sodium acetate) was added. Sensitization was done for 2 h with gentle shaking. Gel was washed with distilled water six times for 15 min each. Approximately, 250 ml silver solution (silver nitrate 0.25 % (w/v) and 0.0004% of formaldehyde 37-42% (v/v) was added to the gel and kept shaking for 2 h. Gel was then washed with water four times in distilled water for 1 min each. After washing gel was developed by adding 250 ml developing solution (2.5 % sodium carbonate, 0.0008% of formaldehyde 37-42% v/v) for 4-6 min and transferred to stopping solution containing 1.4 % EDTA-Na2,2H2O for 2 h once the protein spots had reached desired intensity.
3.5.3.2. Colloidal Coomassie Brilliant Blue Staining

After SDS-PAGE the gel was rinsed twice with 100 ml distilled water for 3 min. Colloidal coomassie (Appendix) blue working solution was added to gel and stained overnight. When the spots became visible the gel was destained with distilled water until the background became clear (Neuhoff et al., 1988).

3.5.4.1. Protein identification and MS analysis

The spots of interest were picked manually from the corresponding coomassie stained gels. For this, a preparative gel was run in which a total of 500 µg of protein was used for rehydrating single strip and IEF and SDS-PAGE were performed in the same conditions as described earlier for the CyDye labeled gels. Corresponding spots from preparative gel were manually picked and destained with 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate solution (NH₄HCO₃). The destained spots were then treated sequentially with 100 µl of 100 mM NH₄HCO₃ and ACN for two cycles and finally dehydrated gel pieces were dried completely in the speed vac to remove any residual acetonitrile.

The gel pieces were then immersed in a digestion buffer (25 mM NH₄HCO₃) containing 10 ng/µl of trypsin on ice. After 45 min of incubation, swollen gel pieces were covered with 50 µl of digestion buffer and incubated overnight at 37°C. Next day, the peptides were extracted from the gel pieces with 50% ACN and 5% FA (Formic acid) and lyophilized peptides then were reconstituted in 20 µl of 50% ACN and 0.1% FA for analysis by ESI-Q-TOF-MS/MS.

3.5.4.2. MS/MS analysis

Digested peptides were solubilized in 50% ACN containing 0.1% FA and loaded in a silica capillary (Proxeon Biosystem, USA) and fixed to a QSTAR XL quadrupole/time-of-flight tandem mass spectrometer (Applied Biosystem, USA). Nanospray ionization was carried out using an ion spray voltage of 900. The progress of each run was monitored by recording the total ion current (TIC) for positive ions as a function of time for ions in the m/z range of 400-1600 for MS and 140-1600 for MS/MS. Mass spectra were acquired for 10 min setting the parameters by information-dependent acquisition (IDA) method. The other parameters set were: interface temperature, 50°C;
curtain gas flow, 1.13 L/min; declustering potential, 60 V; focusing potential, 280 V; declustering potential 2, 15 V.

3.5.5. Database searches

Database searching was done using Mascot (Version 1. Matrix Science, UK). Some modifications were considered such as oxidation of methionine and carbamidomethylation of cysteine while searching. Search was further refined to include peptides with charged state from +2 to +3 and limited to *M. tuberculosis* complex. The peptide mass tolerance range was ± 1.6 kDa and fragment mass tolerance was ± 0.8 kDa. Hits with a probability based Mowse score more than the designated value were considered successfully identified.

**Flow chart illustrating the overall schemes is shown in Figure 3.3.**

Figure 3.3 Schematic of 2-D DIGE experiment. A normalization pool comprising all the samples from condition 1 and Condition 2 is labeled with Cy2 dye. Each 2-D DIGE gel comprises normalization pool (Cy2); Condition 1 (Cy3/Cy5) and Condition 2 (Cy3/Cy5) samples. The three scanned images from each 2-D DIGE and further analyzed by DeCyder Image analysis to generate the differentially regulated protein spots, are further processed by MS. The mass list from the mass spectra is used for peptide mass fingerprinting for protein identification.
3.6. Methods for expression analysis of \textit{rpf} genes in different physiological conditions

3.6.1. Design of reporter constructs to evaluate the expression of \textit{rpf} genes in different physiological conditions

To evaluate and analyze the expression of \textit{rpf} genes in different physiological conditions like nutrient starvation, pH stress and hypoxic conditions, 5’upstream regions of \textit{rpf} genes from \textit{M. tuberculosis} H37Rv were cloned in pLL192 vector (Srivastava \textit{et al.}, 2007). The map of pLL192 is shown in Figure 3.2.

![Schematic representation of promoter probe pLL192 vector](image)

Figure 3.2 Schematic representation of promoter probe pLL192 vector used for promoter cloning and expression of reporter genes encoding green fluorescence protein and kanamycin resistance (Srivastava \textit{et al.}, 2007).
3.6.1.1. Amplification of 5' upstream region *rpF* genes

The 990 bp upstream regions of both *rpFC* and *rpFE*, were amplified by PCR using genomic DNA of *M. tuberculosis* H37Rv as template DNA. The primers and the conditions used for PCR are as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>65°C</td>
<td>1 min</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

The amplified products were of 990 bp (*rpFC* and *rpFE*).

3.6.1.2. Construction of pLL192 reporter constructs

pLL192 is a promoter probe shuttle vector constructed in our lab (Srivastava et al., 2007). The vector has a transcriptional fusion of *gfp* and kanamycin resistance genes as a reporter system downstream of a unique *BamHI* site for cloning of promoter or upstream region. It also contains streptomycin resistance cassette as a selection marker.

The amplified fragment of *rpFC* and *rpFE* were first cloned in TOPO 2.1 and sequenced using M13F and M13R primers. All fragments were excised from the vector by digestion with *BamHI* for *rpFC* and *BglII* for *rpFE* and then cloned into pLL192 vector at the *BamHI* site. The clones were selected for right oriented clone by restriction digestion. The right oriented clone of *rpFC* was selected by digestion with *NruI* and *rpFE* was selected using *PvuII* restriction enzyme. The constructs were electroporated in *M. bovis* BCG and the selection for recombinant clones was made on MB7H10 plates supplemented with OADC containing streptomycin (15 µg/ml). The recombinant clones were further confirmed by colony PCR. A similar construct with constitutive *hsp60* promoter at *BamHI* site served as positive control and the vector alone was used as negative control for all comparative studies.
3.6.2. Different physiological condition used for promoter analysis

The expression of rpf genes was evaluated under, different stress conditions. First, stress condition was hypoxia. The recombinant M. bovis BCG including positive and negative controls were grown in hypoxic condition.

To generate hypoxic condition, the cultures were grown in Sauton’s medium and the seed culture were diluted to $A_{600}$ of 0.05, an oxygen indicator methylene blue (1.5 µg/ml) and 15 µg/ml of streptomycin was added. The culture was subjected to anaerobic condition in sealed tubes with a head space ratio of 0.5 (0.5 HSR) and agitated at a stir rate of 100 rpm with non-detectable perturbations of the surface of the medium, incubated at 37°C in incubator. The culture started decolorizing and complete decolorization was obtained after 21-26 days indicating hypoxia (Saxena et al., 2008).

The second stress condition implemented was nutrient starvation. Cultures were grown in Sauton’s medium in presence of 15 µg/ml of streptomycin. When the $A_{600}$ of the culture reaches to 0.6-0.8, the cells were harvested by centrifugation at 6000 x g for 15 min. The cultures were washed twice with PBS (pH 7.2) and finally suspended in 200 µl PBS. These cells were used for Flow Cytometric analysis at 3rd, 6th, and 12th day of inoculation.

The third condition was related to pH stress in which mycobacteria was subjected to acidic condition. The cultures were first grown in Sauton’s medium till $A_{600}$ became 0.6-0.8. Cultures at this stage were harvested by centrifugation and washed twice with PBS. The pellet was then suspended in 2 ml of PBS. The suspended cultures were finally aliquoted in three parts, and subjected to pH of 4.5, 5.5, and 7.2. The cultures were then agitated at 250 rpm at 37°C for 4 h. The cells were again harvested by centrifugation at 6000 x g for 15 min. The cells were washed twice with PBS and finally suspended in PBS. The cells were taken for Flow Cytometric analysis.

3.6.3. Flow Cytometric Analysis

Flow cytometry was carried out using a FACS caliber system (Becton Dickinson) and the cell quest software provided by the supplier. Excitation was with 200 mW 488 nm argon-laser and emission light was detected through a 520/30 nm band pass filter. Data were collected on $1 \times 10^4$ individual particle per sample.
Flow analysis of *M. bovis* BCG cultures containing promoter reporter constructs in all stress conditions was done by suspending bacilli in PBS. Cells of all the three conditions were harvested by centrifugation and washed twice with PBS (pH 7.2). Cells were then finally resuspended in PBS and measured for GFP (green fluorescent protein) fluorescence intensity and the data was plotted on FSC-FLI (gfp) scale. Appropriate controls including negative (*M. bovis* BCG containing pLL192 plasmid) and positive controls (*M. bovis* BCG containing pLL192 driven by *Phsp60* promoter) were taken during each experiment. The bacterial cells were first gated to separate them from debris and large clumps using dot blot analysis. This was done on FSC and SSC parameters where the internal complexity and size determines the separation of population. FSC separates the population on the basis of their size using forward scatter. SSC is side scatter which separates the population on the basis of their internal complexity. This specific population was then analysed for fluorescence intensity. The results were expressed as percent population fluorescing and the mean fluorescence.

### 3.6.4. Validation of expression of *rpf* genes by Real Time-PCR

#### 3.6.4.1. RNA isolation and Real Time-PCR

RNA was isolated from cultures grown in hypoxia, nutrient starvation and acidic condition using Qiagen RNAeasy kit described above. *M. bovis* BCG culture grown in Sauton's medium in hypoxic condition as described earlier was used for RNA isolation after 6 days of hypoxia. RNA was isolated after 12 days of nutrient starvation from *M. bovis* BCG as described above. Similarly, culture grown in Sauton's medium at pH 7.2, 4.5 and 5.5 was used for isolation of RNA. For Real Time-PCR analysis, each RNA sample was treated with Qiagen RNase-Free DNase and heat-inactivated according to the manufacturer's instructions. 300-400 ng RNA was used for cDNA synthesis using random hexamer primers by Revert Aid First Strand cDNA Synthesis Kit (Fermentas). Real-time quantitative RT-PCR was done using LightCycler RNA Master SYBR Green Kit and Light Cycler 480II (Roche) instrument. Primers used in real-time PCR are described above. All primer sets were tested prior to use to ensure that only a single product of the correct size was amplified by PCR. RT-PCR conditions were as described in the manufacturer's protocol for the RNA Master SYBR Green kit. Negative controls consisting of no-template (water) reaction mixtures were run with all reactions. In each set
of reactions, SigA was used as a reference gene for normalization. Each reaction was repeated three times with three independent RNA samples. Relative Quantification study was done using ΔΔCT method assuming that the PCR efficiencies were the same as seen from the slope of the reaction curves (Livak and Schmittgen, 2001). Melting curve analysis was done for each reaction product to ensure the specificity of amplified product. The results were expressed as fold induction of expression of genes used in analysis.