Chapter 2
MATERIALS AND METHODS

2.1 Materials ................................................................. 28-43
  2.1.1 Bacterial strains .................................................. 28
  2.1.2 Plasmids ........................................................... 28-29
  2.1.3 Primers ............................................................ 29
  2.1.4 Chemicals ......................................................... 29-30
  2.1.5 Antibiotics ........................................................ 30
  2.1.6 Media .............................................................. 30-32
  2.1.7 Antibodies ......................................................... 32-33
  2.1.8 Buffers and solutions for DNA work ................................... 33-37
  2.1.9 Buffers and solutions for RNA work .................................... 38-39
  2.1.10 Buffers and solutions for detection and analysis of protein ............. 39-42
  2.1.11 Buffers and solutions for protein purification and assay ............... 42-43

2.2 Methods ................................................................. 43-56
  2.2.1 Isolation of plasmid DNA .......................................... 43-44
  2.2.2 Genomic DNA isolation from mycobacterial cultures ....................... 44-45
  2.2.3 RNA isolation from E. coli ........................................ 45
  2.2.4 Nucleic acid quantitation ......................................... 45-46
  2.2.5 Agarose gel electrophoresis ........................................ 46
  2.2.6 Restriction endonuclease digestion ................................ 46
  2.2.7 Purification of DNA fragment from agarose gels .......................... 47
  2.2.8 Nucleic acid manipulation ......................................... 47
  2.2.9 Preparation of competent cells and transformation in E. coli .............. 47-48
  2.2.10 Polymerase chain reaction ....................................... 48-49
  2.2.11 DNA sequencing and analysis ..................................... 49
  2.2.12 Southern/Northern blotting and hybridization .......................... 49-51
  2.2.13 Site-directed mutagenesis ....................................... 51
  2.2.14 Protein estimation ............................................... 51
  2.2.15 SDS-PAGE ....................................................... 51-52
  2.2.16 Expression and purification of recombinant proteins ..................... 52
  2.2.17 Western blotting ................................................ 53
  2.2.18 ELISA ............................................................ 53-54
  2.2.19 Kinase and dephosphorylation assays ................................ 54
  2.2.20 In vitro interaction studies with E. coli lysate ........................ 54-55
  2.2.21 Phosphatase assay .............................................. 55
  2.2.22 Scanning electron microscopy .................................... 55
  2.2.23 Bioinformatic analysis .......................................... 55-56
  2.2.24 Data analysis .................................................. 56
2.1 Materials

2.1.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. *Escherichia coli* was either grown in LB or superbroth and was maintained on LB agar plates. *Mycobacterium* species were grown either in liquid Sauton’s medium (Sauton, 1912) or Middlebrook 7H9 supplemented with or without 0.01% Tween 80. Middlebrook 7H10 agar plates or LJ slants was used as solid medium to cultivate mycobacteria. Medium was supplemented with ADC or OADC enrichment wherever necessary. All the bacteria were grown at 37°C (with shaking at 200 rpm for liquid cultures). For routine storage, plates were maintained at 4°C and for long term storage, cultures were kept in 15% glycerol at -70°C.

2.1.2 Plasmids

The following vectors have been used in this study:

a) **pUC19**: a pBR322 derived high copy number plasmid. It carries ampicillin resistance marker, *lacZ*, extensive multiple cloning site and ColE1 compatibility group origin of replication (Norrander *et al.*, 1983).

b) **pGEM-7Zf(+)**: pUC derived plasmid containing ampicillin resistance gene as marker. It has an extensive polylinker cloning site with *lacZ* (Yanisch-Perron *et al.*, 1985).

c) **pKK223-3**: carries pBR322 origin of replication and ampicillin resistance marker. The vector is used for the constitutive expression of proteins in *E. coli* under the control of tac promoter (Brosius and Holy, 1984).

d) **pET-23a**: a pBR322 derived plasmid. It is an *E. coli* expression vector containing T7 promoter and ampicillin resistance gene as selection marker (Studier, 1991).

e) **pET-28c**: a pBR322 derived plasmid. It is an *E. coli* expression vector containing T7lac promoter and kanamycin resistance marker (Studier, 1991).

f) **pQE-30**: belongs to the pDS family of plasmids (Bujard *et al.*, 1987) and derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (Stueber *et al.*...
# Table 2.1 Bacterial strains used in the study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype or phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>F'ompT [lon] hsdS&lt;sub&gt;B&lt;/sub&gt; (rB&lt;sup&gt;+&lt;/sup&gt;mB&lt;sup&gt;-&lt;/sup&gt;; an <em>E. coli</em> B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene, has lac UV5 promoter inducible by IPTG</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; φ80lacZΔM15 endA1 recA1 hsdR17 (r&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;+&lt;/sup&gt;) supE44 thi-1, gyrA96 relA1 Δ(lacZYA-argF)U169 λ&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em> M15</td>
<td>Derived from <em>E. coli</em> K12 and have the phenotype Na&lt;sup&gt;+&lt;/sup&gt;, Str&lt;sup&gt;−&lt;/sup&gt;, Rif&lt;sup&gt;−&lt;/sup&gt;, Thi&lt;sup&gt;−&lt;/sup&gt;, Lac&lt;sup&gt;−&lt;/sup&gt;, Ara&lt;sup&gt;−&lt;/sup&gt;, Gal&lt;sup&gt;−&lt;/sup&gt;, Mtl&lt;sup&gt;−&lt;/sup&gt;, F&lt;sup&gt;−&lt;/sup&gt;, RecA&lt;sup&gt;+&lt;/sup&gt;, Uvr&lt;sup&gt;+&lt;/sup&gt;, Lon&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em> SG13009</td>
<td>Derived from <em>E. coli</em> K12 and have the phenotype Na&lt;sup&gt;−&lt;/sup&gt;, Str&lt;sup&gt;−&lt;/sup&gt;, Rif&lt;sup&gt;−&lt;/sup&gt;, Thi&lt;sup&gt;−&lt;/sup&gt;, Lac&lt;sup&gt;−&lt;/sup&gt;, Ara&lt;sup&gt;−&lt;/sup&gt;, Gal&lt;sup&gt;−&lt;/sup&gt;, Mtl&lt;sup&gt;−&lt;/sup&gt;, F&lt;sup&gt;−&lt;/sup&gt;, RecA&lt;sup&gt;+&lt;/sup&gt;, Uvr&lt;sup&gt;+&lt;/sup&gt;, Lon&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em> TB1</td>
<td>ara Δ(lac proAB), rpsL (φ80 lacZΔM15) hsdR</td>
</tr>
<tr>
<td><em>E. coli</em> XL1 Blue</td>
<td>hsdR17 recA1 lacF&lt;sup&gt;−&lt;/sup&gt; [proAB&lt;sup&gt;+&lt;/sup&gt;lacI&lt;sup&gt;+&lt;/sup&gt;lacZ Δ15 Tn 10 (tet&lt;sup&gt;−&lt;/sup&gt;)]</td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc&lt;sup&gt;2&lt;/sup&gt; 155</td>
<td>Fast growing (doubling time ~3 h) saprophytic high frequency derivative of <em>M. smegmatis</em> ATCC 607</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG ATCC 35734</td>
<td>Non-pathogenic laboratory strain of <em>M. bovis</em></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Ra NCTC 7417</td>
<td>Avirulent strain of <em>M. tuberculosis</em></td>
</tr>
</tbody>
</table>
al., 1990). It carries T5 promoter, ampicillin resistance marker and ColE1 origin of replication. The vector is used for the expression of 6x His-tagged proteins in *E. coli*.

g) **pGEX-KG**: derivative of pGEX-2T, used for the expression of glutathione-S-transferase (GST) fusion proteins in *E. coli*. It carries tac promoter, lac repressor gene, ampicillin resistance marker and pBR322 origin of replication (Guan and Dixon, 1991).

h) **pMAL-c2**: used for the cytoplasmic expression of proteins in *E. coli* in fusion with maltose binding protein (MBP) encoded by *malE*. It carries tac promoter, lac repressor gene, ampicillin resistance marker, pBR322 and M13 origins of replication (Maina et al., 1988).

i) **p19Kpro**: *Mycobacterium - E. coli* shuttle vector for the constitutive expression of genes using *M. tuberculosis* 19 kDa antigen promoter. It carries hygromycin resistance gene as selection marker (Garbe et al., 1994). The plasmid was obtained as a kind gift from Drs. D. B. Young and M. Blokpoel, Imperial College School of Medicine at St. Mary’s, London, UK.

### 2.1.3 Primers

Oligonucleotide primers used in this work are listed in Tables 2.2 and 2.3. The primers were commercially synthesized from Ransom Hill Bioscience Inc., California, USA or Integrated DNA Technologies Inc., Coralville, IN, USA or Biobasic Inc., Canada. Besides these, other sequencing primers were procured from commercial sources (Table 2.4).

### 2.1.4 Chemicals

All reagents used in this study were of analytical grade and were obtained from commercial sources. Restriction/modifying enzymes, molecular biological reagents and amylose resin were obtained from New England Biolabs. ECL Plus Western blotting detection kit (Amersham Pharmacia, England), Expand Long Template PCR system (Roche Applied Science, Germany), X-ray film (Eastman Kodak, USA) and nylon/nitrocellulose membranes (Bio-Rad, USA or Amersham
Table 2.2  PCR primers for pknA

<table>
<thead>
<tr>
<th>a) Amplification of <em>M. tuberculosis</em> pknA:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC7 (sense) 5' CATATGAGCCCCCGAGTTGG 3'</td>
</tr>
<tr>
<td>CC8 (antisense) 5' TCATTGCCTATCTCGTATCGG 3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Point mutants of pknA:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External primers</strong></td>
</tr>
<tr>
<td>CC58 (sense) 5' CACAGGAATTCCATATGAGCCCCCGAGTTGG 3'</td>
</tr>
<tr>
<td>CC61 (antisense) 5' CTGCCCGGTGGGGGTGATCAAGATG 3'</td>
</tr>
<tr>
<td><strong>Internal primers</strong></td>
</tr>
<tr>
<td>CC62 (sense) 5' GTGTTGCGGTGAATTGTGCTCAAGAGCG 3'</td>
</tr>
<tr>
<td>CC63 (antisense) 5' CGCTCTTGGAGCACATTCACCGCAACAC 3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c) Deletion mutants of pknA:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC13 (sense) 5' CAACAGTATAGAATTCGTAAGAAGAACCCCGCGATG 3'</td>
</tr>
<tr>
<td>CC14 (antisense) 5' CAACAGTATAGACCTATTCGCAAGCAGTTATCGG 3'</td>
</tr>
<tr>
<td>CC58 (sense) 5' CACAGGAATTCCATATGAGCCCCCGAGTTGG 3'</td>
</tr>
<tr>
<td>CC68 (antisense) 5' CACCCAAGCTTTGCAGCGAAGCGTCCCCACCAC 3'</td>
</tr>
<tr>
<td>CC69 (antisense) 5' CACCCAAGCTTTCAACGCTGACCGGACGAAAAC 3'</td>
</tr>
</tbody>
</table>

* Underlined bases represent base mismatches for desired mutations
Table 2.3  PCR primers for \textit{ppp}

\begin{tabular}{|l|l|}
\hline
a)  & \textbf{Amplification of \textit{M. tuberculosis} \textit{ppp}:} \\
CC39 (sense) & 5' CATATGGCGCGTGACCCTG 3' \\
CC34 (antisense) & 5' TCGTCATGCGCCGCCC 3' \\
\hline
b)  & \textbf{Sequencing of \textit{ppp}:} \\
CC35 (sense) & 5' CCTGCCCAACACCGCCGC 3' \\
CC37 (sense) & 5' CTGACGCAGATCAACACAAGGAC 3' \\
\hline
c)  & \textbf{Point mutants of \textit{ppp}:} \\
\textbf{External primers} & \\
CC76 (sense) & 5'CACAGGAATTCCATATGGCGCGTGACCCTG 3' \\
CC77 (antisense) & 5' CAGTGACGTTTGTCGGGCGCCGCC 3' \\
\textbf{Internal primers} & \\
CC70 (antisense) & 5'AGACCGAGTCTTGGTTGCGC 3' \\
CC71 (sense) & 5' CGCAAAACCGCAGAAGACTCCGTCT 3' \\
CC72 (antisense) & 5' CCATGCCGCGGCCAGG 3' \\
CC73 (sense) & 5' CCTGGCGGCCGGCATGG 3' \\
CC74 (antisense) & 5' CGCGCGAGTGATCGATGATGACCC 3' \\
CC75 (sense) & 5' GGTGCATATCGATCGATCGCGC 3' \\
\hline
\end{tabular}

- Underlined bases represent base mismatches for desired mutations
### Table 2.4  Sequencing primers

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 universal:</td>
<td>5' GTAAAACGACGGCCAGT 3'</td>
</tr>
<tr>
<td>M13 reverse:</td>
<td>5' CAGGAAACAGCTATGAC 3'</td>
</tr>
<tr>
<td>pGEX 5':</td>
<td>5' GGGCTGGCAAGCCACGTTTGTG 3'</td>
</tr>
<tr>
<td>pGEX 3':</td>
<td>5' CCGGGAGCTGCATGTGTCAGAGG 3'</td>
</tr>
<tr>
<td>MalE primer:</td>
<td>5' GGTCGTCAGACTGATGAAGCC 3'</td>
</tr>
</tbody>
</table>
Pharmacia, England or Advanced Microdevices, India) were commercially available. Protein molecular weight markers were purchased from Bio-Rad, New England Biolabs or Sigma Chemical Company, USA. Plasmid isolation, gel extraction and PCR purification kits used in this study were obtained from Qiagen, Germany. All other chemicals including Bradford reagent, casein, histone, leupeptin, maltose, myelin basic protein, N-lauroyl sarcosine (sarkosyl), okadaic acid, pepstatin, phenylmethylsulfonyl fluoride (PMSF), p-nitrophenyl phosphate (pNPP), sodium orthovanadate etc., were procured from Sigma Chemical Company, USA. \( [\gamma^{32}\text{P}] \)-ATP and \( [\alpha^{32}\text{P}] \)-CTP with specific activity of 3000 - 5000 Ci/m mole were procured from Jonaki Laboratories, Board Radiation and Isotope Technology, Hyderabad, India.

2.1.5 Antibiotics

Ampicillin, hygromycin, kanamycin and tetracycline used in this study were procured from commercial sources (Roche Applied Science, Germany and Sigma Chemical Company, USA) and their stocks were prepared as follows:

Ampicillin: 100 mg/ml in water
Kanamycin: 25 mg/ml in water
Tetracycline: 12.5 mg/ml in 70% ethanol
Hygromycin: 50 mg/ml in phosphate buffered saline (PBS)

Stock solutions of antibiotics were sterilized through 0.22 \( \mu \text{M} \) filters (Millipore) and stored in aliquots at -20°C.

2.1.6 Media

Media were prepared using distilled water and were autoclaved for 15 min at 121°C at 15 pounds per square inch pressure. Stock solutions of glucose, non-autoclavable drugs and chemicals such as isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG) were sterilized by filtration through 0.22 \( \mu \text{M} \) filters (Millipore) before adding to media. Unless mentioned otherwise, media were prepared following procedures as described in Sambrook et al. (1989); Sambrook and Russel (2001).
**LB medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
</tbody>
</table>

The above contents were dissolved in 900 ml water and pH was adjusted to 7.5 with 10 N NaOH. Final volume was made to 1 L with water.

LB agar contained 1.5% agar in LB medium.

LB medium was cooled down to ~45°C prior to the addition of antibiotics.

**Super Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>32 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>20 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
</tbody>
</table>

The above contents were dissolved in 900 ml water and pH was adjusted to 7.5 with 10 N NaOH. Final volume was made to 1 L with water.

**Sauton’s medium** (Sauton, 1912)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Magnesium sulfate (MgSO₄.7H₂O)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Asparagine</td>
<td>4.0 g</td>
</tr>
</tbody>
</table>

Contents were dissolved in 600 ml water by heating up to 70°C. 60 ml glycerol was added after adjusting the pH to 7.2 with 10 N NaOH. Volume was made to 1 L by the addition of water. Medium was supplemented with 0.01% Tween 80.

**Middlebrook 7H9 medium**

Obtained from Difco and used according to manufacturer’s recommended protocol. Middlebrook ADC/OADC enrichment and 0.01% Tween 80 were added to the sterile media just before inoculation.
Middlebrook 7H10 agar
Obtained from Difco and used according to manufacturer’s protocol. Middlebrook OADC enrichment was added to autoclaved media as per manufacturer’s instructions.

Lowenstein Jensen (LJ) slants
3.73 g of Lowenstein Jensen medium base (Himedia) was suspended in 60 ml of double distilled water containing 1.25 ml glycerol. The contents were dissolved by boiling and were autoclaved at 121°C, 15 pounds per square inch pressure for 15 min. Three eggs were washed and scrubbed with soapy water, rinsed in running water for at least 30 min and dried after wiping with alcohol. All subsequent steps were carried out in the laminar hood. The eggs were cracked open with a knife in a sterile beaker. These were beaten to prepare homogeneous emulsion (around 100 ml) and filtered through three layers of autoclaved gauze. The contents were mixed with LJ base and ~8 - 10 ml was suspended in each vial. After screwing the caps, the vials were kept tilted horizontally with the help of a wooden rod.

X-gal indicator plates
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) indicator plates were made to screen the recombinant clones by blue-white screening. 50 µl of X-gal (20 mg/ml in dimethyl formamide) and 25 µl of IPTG (20 mg/ml in water) were plated on LB agar plates containing appropriate antibiotics.

2.1.7 Antibodies
The following antibodies were used during the course of this work:

a) Primary antibodies
i. Anti-GST antibody: monoclonal antibody from mouse. It was purchased from Roche Applied Science, Germany and used at a dilution of 1:1000.
ii. Anti-MBP serum: rabbit serum prepared using purified maltose binding protein was obtained from New England Biolabs, USA and was used at a dilution of 1:5000.
iii. **Anti-phosphoserine antibody:** monoclonal antibody from mouse raised against phosphoserine conjugated to KLH (Clone PSR-45) was purchased from Sigma Chemical Company, USA and was used at a final dilution of 1:500.

iv. **Anti-phosphothreonine antibody:** polyclonal antibody produced by immunizing rabbit with a phosphothreonine peptide was procured from New England Biolabs, USA and used at a dilution of 1:1000.

v. **Anti-phosphotyrosine antibody:** PY99, mouse monoclonal IgG from Santa Cruz Biotechnology Inc., USA, used at a dilution of 1:1000.

b) **Secondary antibodies**

i. **Anti-mouse IgG-HRP:** polyclonal antibody raised against mouse IgG heavy and light chains and conjugated to horseradish peroxidase (HRP). It was procured from Roche Applied Science, Germany and used at a dilution of 1:3000.

ii. **Anti-rabbit IgG-HRP:** antibody against IgG fractions from normal rabbit serum, raised in donkey and conjugated to HRP was purchased from Amersham Pharmacia, England and used at a final dilution of 1:5000.

### 2.1.8 Buffers and solutions for DNA work

A. **Basic solutions**

**6x DNA gel loading buffer**

- Bromophenol blue: 0.25%
- Glycerol: 30.0%
- In deionized water.
**50x TAE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Deionized water to make final volume to 1 L.

**TE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris. Cl (pH 8.0)</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

In deionized water.

**Phenol**

Distilled phenol was equilibrated once with 0.5 M Tris. Cl, pH 8.0 and repeatedly with 0.1 M Tris. Cl, pH 8.0 till the pH of phenol reached >7.6. Hydroxyquinoline was added to the equilibrated phenol at a final concentration of 0.1%. The phenol solution was stored at 4°C in an amber coloured bottle to prevent exposure to light.

**B. Alkaline lysis solutions**

**STE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Tris. Cl (pH 8.0)</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

Prepared in deionized water.

**Solution I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris. Cl (pH 8.0)</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

In deionized water.
Solution II
NaOH 0.2 N
SDS 1.0%
In deionized water.

Solution III
5 M Potassium acetate 60.0 ml
Glacial acetic acid 11.5 ml
Deionized water 28.5 ml

C. Solutions for mycobacterial genomic DNA isolation

10x cell wall inhibition solution for *M. smegmatis* mc^2^155
\[\text{α-cycloserine} \quad 600 \, \mu g/ml\]
\[\text{EDTA} \quad 20 \, \text{mg/ml}\]
\[\text{Lysozyme} \quad 2 \, \text{mg/ml}\]
\[\text{Glycine} \quad 140 \, \text{mg/ml}\]
Prepared in deionized water.
The solution was used at a final concentration of 1x.

10x cell wall inhibition solution for *M. tuberculosis* H37Ra and *M. bovis* BCG

Glycine 15%
Lysozyme 2 mg/ml
EDTA 20 mg/ml
D-cycloserine 10 mg/ml
Lithium chloride 10 mg/ml
Prepared in deionized water.
The solution was used at a final concentration of 1x.
Mycobacterial spheroplasting buffer

Tris. Cl (pH 8.0) 50 mM
EDTA 5 mM
Sucrose 20%
Lysozyme 1-2 mg/ml
Prepared in deionized water.

10% SDS Prepared in deionized water.
Proteinase K 20 mg/ml in deionized water.

D. Solutions for Southern blotting and hybridization

Denaturation buffer

NaOH 0.5 M
NaCl 1.5 M
Prepared in deionized water.

Neutralization buffer

Tris. Cl (pH 7.4) 0.5 M
NaCl 1.5 M
Prepared in deionized water.

50x Denhardt's reagent

Ficoll 10 g
Polyvinylpyrrolidone 10 g
Bovine serum albumin 10 g
Deionized water to make final volume to 1 L.
Salmon sperm DNA (ssDNA)

210 mg ssDNA was dissolved in 21 ml water by keeping it at 65°C for 2 h followed by incubation at 37°C. The suspension was boiled for 15 min and then passed through 20 G syringe. Aliquots were made and stored at -20°C.

20x SSC

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.3 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>88.2 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td></td>
</tr>
</tbody>
</table>

Deionized water to make final volume to 1 L.

Prehybridization/hybridization buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>50.0%</td>
</tr>
<tr>
<td>SSC</td>
<td>6x</td>
</tr>
<tr>
<td>Denhardt's reagent</td>
<td>5x</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
</tr>
<tr>
<td>ssDNA</td>
<td>100 μg/ml</td>
</tr>
</tbody>
</table>

In deionized water.

ssDNA was boiled for 15 min and chilled rapidly on ice prior to its addition to the buffer.

Oligo hybridization buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>6x</td>
</tr>
<tr>
<td>Sodium phosphate buffer (pH 6.8)</td>
<td>0.01 M</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>1 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
</tr>
<tr>
<td>ssDNA</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Non-fat milk</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

In deionized water.

Non-fat milk and ssDNA were added just before use. ssDNA was added after boiling it for 15 min and then rapidly chilling on ice.
2.1.9 Buffers and solutions for RNA work

A. Basic solutions

5x Formaldehyde gel running buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>100 mM</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>40 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

Prepared in DEPC treated autoclaved water.
pH was adjusted to 7.0.

10x Formaldehyde gel loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>50%</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>1 mM</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.25%</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

In DEPC treated autoclaved water.

B. Solutions for RNA isolation by hot phenol method

AE buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 M Sodium acetate (pH 5.3)</td>
<td>5 ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8.0)</td>
<td>6 ml</td>
</tr>
</tbody>
</table>

Volume made to 300 ml with DEPC treated autoclaved water.

AE saturated phenol

Mixed equal volumes of distilled phenol and AE buffer in an autoclaved bottle. Stirred overnight for equilibration.
Ice-isopropanol bath

80% isopropanol solution in water. Kept at -70°C for 3 - 5 h to form slush.

10% SDS

In DEPC treated autoclaved water.

10x DNase I digestion buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris. Cl (pH 8.0)</td>
<td>20 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

In DEPC treated autoclaved water.

C. Solutions for Northern blotting and hybridization

All the solutions were same as used for Southern blotting and hybridization except that denaturation and neutralization buffers were not required.

2.1.10 Buffers and solutions for detection and analysis of protein

A. Solutions for SDS-PAGE

Acrylamide (30%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30 g</td>
</tr>
<tr>
<td>N, N'-Methylene bisacrylamide</td>
<td>0.8 g</td>
</tr>
</tbody>
</table>

Made up to 100 ml with deionized water.

Lower Tris (pH 8.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>18.17 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

Made to 100 ml with deionized water after adjusting pH 8.8 with 6 N HCl.
Upper Tris (pH 6.8)

Tris 6.06 g
10% SDS 4 ml

Made to 100 ml with deionized water after adjusting pH 6.8 with 6 N HCl.

5x Sample buffer

Tris. Cl (pH 6.8) 0.15 M
SDS 5%
Glycerol 25%
β-mercaptoethanol 12.5%
Bromophenol blue 0.06%

Volume made to 10 ml with deionized water.

Laemmli buffer (Laemmli, 1970)

Tris 3 g
Glycine 14.4 g
SDS 1 g

Volume made upto 1 L with deionized water.

Gel staining solution

Acetic acid 10%
Methanol 40%
Coomassie brilliant blue R250 0.1%

In deionized water.

Gel destaining solution

Acetic acid 10%
Methanol 40%
Deionized water 50%
The composition of the stacking and resolving gels was same as specified by Sambrook \textit{et al.} (1989); Sambrook and Russel (2001).

B. \textbf{Solutions for ELISA and Western blotting}

\textbf{Transfer buffer}

- Glycine: 39 mM
- Tris: 48 mM
- SDS: 0.037%
- Methanol: 20%

In deionized water.

pH was adjusted to 8.3 before adding methanol.

\textbf{Ponceau S solution}

- Ponceau S: 0.1 g
- Glacial acetic acid: 5 ml

Deionized water to a final volume of 100 ml.

\textbf{Tris buffered saline (TBS), pH 7.5}

- Tris: 50 mM
- NaCl: 150 mM

In deionized water.

pH adjusted to 7.5 with 6 N HCl.

\textbf{TBST}

0.1% Tween 20 in TBS.

\textbf{Phosphate buffered saline (PBS), pH 7.4}

- NaCl: 80 g
- KCl: 2 g
- Na$_2$HPO$_4$.2H$_2$O: 12 g
KH₂PO₄ 2.1 g
Dissolved in 1 L deionized water for 10x stock.
Diluted to 1x with deionized water and pH adjusted to 7.4.

PBST
0.05% Tween 20 in PBS.

Blocking agent
5% Skimmed milk or Bovine serum albumin (BSA) in TBST or PBST.

0.05 M Carbonate buffer (pH 9.6)
Mixed 16 ml of 0.2 M Na₂CO₃ and 34 ml of 0.2 M NaHCO₃ so that final pH was 9.6. Diluted it four times before use.

2.1.11 Buffers and solutions for protein purification and assay

Lysis buffer (TEN buffer)
Tris. Cl (pH 7.5) 20 mM
NaCl 200 mM
EDTA 1 mM
Pepstatin 1 µg/ml
Leupeptin 1 µg/ml
PMSF 0.15 mM

Elution buffer
Tris. Cl (pH 7.5) 20 mM
NaCl 200 mM
EDTA 1 mM
Maltose 10 mM
Kinase buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris. Cl (pH 7.5)</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

2.2 METHODS

2.2.1 Isolation of plasmid DNA

The rapid alkaline lysis method of plasmid isolation described by Sambrook et al. (1989); Sambrook and Russel (2001) was followed. For small scale preparation of plasmid DNA, 1.5 ml of *E. coli* culture was grown to saturation. Cells were pelleted and washed once with STE buffer followed by suspension in 0.1 ml of ice-cold alkaline lysis solution I by vigorous vortexing. The cells were lysed by the addition of 0.2 ml of freshly prepared alkaline lysis solution II. The contents of the tube were mixed gently and stored at room temperature till the lysis was complete. 0.15 ml of ice-cold alkaline lysis solution III was added, contents were mixed by inverting the tube and kept on ice for 10 min. The tube was centrifuged at 9,600xg (4°C for 10 min). The supernatant was transferred to a fresh tube and extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). 0.6 volume of isopropanol was added and mixed well by inverting the tube. The DNA was pelleted by centrifugation at room temperature for 10 min. The pellet was rinsed with 1 ml of 70% ethanol at room temperature, dried and dissolved in TE. RNase treatment was given for 1 h at 37°C at a final concentration of 100 µg/ml. Phenol:chloroform extraction and isopropanol precipitation was again carried out to remove RNase from DNA preparation. The DNA was stored at 4°C until used. Plasmid DNA for sequencing was purified using ABI kit. Briefly, the cell pellet was treated with alkaline lysis solutions. The contents were centrifuged and the supernatant obtained was mixed with binding buffer. The entire solution was poured onto a miniprep column, the resin of which preferentially binds supercoiled plasmid DNA. The resin was washed with wash buffer and the DNA was finally eluted in water.
For large scale plasmid DNA preparation, plasmid miniprep method was scaled up with certain modifications. Cell pellet from 25 ml overnight culture was washed with STE buffer and suspended in alkaline lysis solution I followed by treatment with lysozyme (1 mg/ml) for 15 min at 37°C. After addition of alkaline lysis solution III, the suspension was filtered through glasswool before isopropanol precipitation. Alternatively, commercially available plasmid preparation kits (Qiagen, Germany) based on a modified alkaline lysis procedure were used. The supernatant obtained from the treatment of cell pellet with alkaline lysis solutions was allowed to bind to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins and other impurities were removed by a medium-salt wash. Plasmid DNA was eluted in a high-salt buffer and desalted by isopropanol precipitation.

2.2.2 Genomic DNA isolation from mycobacterial cultures

*M. smegmatis* culture was grown to mid log phase (OD$_{640}$ ~0.6) and processed for the preparation of spheroplasts. For this, the culture was treated with 1x cell wall inhibition solution (Section 2.1.8C) for 16 – 18 h. The resulting wall deficient forms of cells were pelleted at 4,000xg, 4°C for 10 min and incubated with mycobacterial spheroplasting buffer (Section 2.1.8C) at 37°C for 1 h (Rastogi and David, 1981). Spheroplasts thus obtained were lysed by incubating them with 3.5% SDS and proteinase K (250 μg/ml) for 1 h at 37°C. Standard protocols (Sambrook et al., 1989; Sambrook and Russel, 2001) were followed for nucleic acid extraction with a phenol:chloroform mixture (1:1) and DNA pellet was obtained by ethanol precipitation. The pellet was dissolved in TE and RNase digestion was carried out (100 μg/ml at 37°C for 1 h). Finally, DNA was extracted with phenol:chloroform, precipitated with ethanol, dissolved in TE and stored at -20°C.

For genomic DNA isolation from *M. tuberculosis* H37Ra and *M. bovis* BCG, certain modifications were made in the protocol for DNA isolation from *M. smegmatis*. The saturated cultures were treated with 1x cell wall inhibition solution (Section 2.1.8C) at 37°C for 24 h. Wall deficient forms of cells were harvested by centrifugation at 4,000xg for 20 min at 4°C and incubated with mycobacterial
spheroplasting buffer (Section 2.1.8C) at 37°C for 24 h. Incubation was carried out at 37°C for another 24 h after the addition of 4% SDS and 500 µg/ml proteinase K. This was followed by phenol:chloroform extraction, ethanol precipitation and RNase treatment as mentioned above.

2.2.3 RNA isolation from E. coli

Total RNA was isolated from E. coli by hot phenol extraction method (Schmitt et al., 1990). 10 ml cultures were grown in LB medium to an OD$_{600}$ of 0.7 - 0.8. Cells were harvested by centrifugation, washed with water and resuspended in 400 µl of ice-cold AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA). The cells were lysed with 40 µl of 10% SDS aided by vortexing and extracted once with an equal volume of fresh phenol equilibrated with AE buffer. The contents were incubated at 65°C for 4 min and rapidly chilled in ice-isopropanol bath until phenol crystals appeared. Heating and freezing cycle was repeated thrice and the contents were centrifuged at room temperature for 5 min. The upper aqueous phase was transferred to fresh microfuge tube and extracted with phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature for 5 min. RNA in the aqueous phase was precipitated in the presence of 0.3 M sodium acetate, pH 5.3 and 2.5 volumes of ethanol. The pellet was washed with 70% ethanol, dried and suspended in water. This was followed by DNase treatment (1 unit/50 µl reaction) at 37°C for 1 h. Phenol:chloroform extraction and ethanol precipitation was again carried out. Finally, the pellet obtained was dissolved in TE. RNA was checked quantitatively by spectrophotometric analysis and qualitatively by denaturing agarose gel electrophoresis. RNA prepared in this way was processed further for Northern blot.

2.2.4 Nucleic acid quantitation

The DNA/RNA was quantitated spectrophotometrically (Beckman DU 640B spectrophotometer) by measuring absorbance at 260 nm. An absorbance unit of 1 at 260 nm was taken to be equivalent to a concentration of 50 µg/ml double stranded DNA and 40 µg/ml of RNA (Sambrook et al., 1989; Sambrook and Russel, 2001).
The purity of DNA/RNA was confirmed by taking $\text{OD}_{260}/\text{OD}_{280}$ ratio. Purified DNA had an $\text{OD}_{260}/\text{OD}_{280}$ ratio of $\sim 1.8$ whereas purified RNA had a ratio of $\sim 2.0$.

### 2.2.5 Agarose gel electrophoresis

DNA fragments were fractionated on 0.8% agarose gels as a routine. 1% - 2% gels were run for analyzing very small fragments (<500 bp), whereas for restricted chromosomal DNA, 0.6 – 0.7% gels were used. 6x gel loading buffer was added to DNA samples at a final concentration of 1x prior to loading onto the gel. Electrophoresis was carried out in 1x TAE buffer at 10 V/cm for the resolution of restricted plasmid DNA or at 3 - 5 V/cm for chromosomal DNA. Ethidium bromide (0.5 μg/ml) was supplemented in the agarose gel for visualizing DNA on a UV transilluminator (Fotodyne, USA). Lambda DNA fragments generated by *Hind* III digestion, 100 bp DNA ladder and 1 kb DNA ladder were used as molecular size markers for calculating the size of unknown DNA fragments from their relative mobility.

Denaturing agarose gels were run for the qualitative analysis of RNA. The RNA gel apparatus was pretreated with SDS and hydrogen peroxide. 1.2% formaldehyde-agarose gels were cast in running buffer consisting of MOPS, sodium acetate and EDTA and prerun at 5 - 6 V/cm for 10 min. RNA samples were mixed with 50% formamide, 17.5% formaldehyde and 1x gel running buffer, heated at 65°C for 15 min and chilled rapidly on ice. Gel loading buffer at a final concentration of 1x and 0.05 μg/μl ethidium bromide were added before loading the samples.

### 2.2.6 Restriction endonuclease digestion

DNA samples were digested with restriction endonucleases in their specific reaction buffers at desired temperatures for 2 h (New England Biolabs). Reaction mixture was further heated at 75°C for 10 min to inactivate the enzyme. The samples were mixed with DNA gel loading buffer and fractionated on agarose slab gel electrophoresis.
2.2.7 Purification of DNA fragment from agarose gels

After electrophoresis, DNA was visualized using a UV transilluminator in a long wavelength (302 nm). Agarose blocks containing the desired DNA fragment(s) were excised out and weighed. DNA was extracted using a gel extraction kit (Qiagen, Germany). Briefly, 3 volumes of solubilization and binding buffer were added to 1 volume of the agarose gel slice. The contents were incubated at 50°C till complete dissolution of the agarose gel. The suspension was then poured onto a Qiaquick spin column to allow the adsorption of DNA on silica-gel membrane. The impurities were washed away with ethanol-containing buffer. DNA was finally eluted in elution buffer (10 mM Tris. Cl pH 8.5) or autoclaved distilled water and quantitated.

2.2.8 Nucleic acid manipulation

Modifying enzymes such as calf intestinal alkaline phosphatase, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase (Klenow fragment) etc., were used for cloning of DNA fragments according to the instructions provided by Sambrook et al. (1989); Sambrook and Russel (2001).

2.2.9 Preparation of competent cells and transformation in E. coli

Single colony of E. coli was inoculated in LB or superbroth medium and grown to saturation. The culture was reinoculated in fresh medium at a dilution of 1:100 and grown to mid log phase (OD600 of 0.4 - 0.5). The cells were pelleted, chilled on ice for 15 min and processed further for the preparation of competent cells and transformation by any of the following methods.

CaCl2 method: CaCl2 competent E. coli cells were prepared by the method of Cohen et al. (1972). The harvested cells were washed with 0.12 volumes of ice-cold CaCl2 buffer (60 mM CaCl2 and 15% glycerol), suspended in the same buffer and incubated on ice for 30 min. Following centrifugation, the pellet was resuspended in 0.02 volumes of the buffer, 200 µl aliquots were made and stored at -70°C. For transformation, ligation mix (10 µl containing ~100 ng of DNA) was added to an aliquot and incubated on ice for 30 min. Following heat shock treatment at 42°C for
90 sec, the cells were kept on ice for 5 min. Transformed cells were incubated at 37°C for 1 h after the addition of 800 µl LB broth to allow the expression of antibiotic resistant gene(s). Cells were then plated on LB plates supplemented with the desired antibiotics.

**Electroporation:** *E. coli* cells were alternatively transformed by electroporation for which competent cells were prepared according to the method of Dower *et al.* (1988). The cells were washed with equal volume of water followed by washing twice with 0.5 volumes of ice-cold 1 mM HEPES buffer (pH 7.0). Cells were then washed with 0.04 volumes of ice-cold 10% glycerol. Finally, these were suspended in ice-cold 10% glycerol and 50 µl aliquots were made. Ligation mix (1 µl containing ~20 ng DNA) was added to an aliquot and electroporated in 0.2 cm cuvettes, using a Bio-Rad Gene Pulser at a setting of 2.5 KV/cm, 25 µfd and 200 Ω. 1 ml LB broth was added to the transformed cells and incubated at 37°C for 1 h. Cells were plated on LB plates supplemented with desired antibiotics. X-gal and IPTG were included at this step whenever blue-white selection was required.

**2.2.10 Polymerase chain reaction**

PCR amplification was carried out with 100 to 200 ng template DNA in a final reaction volume of 50 µl containing 500 µM of each dNTP (dATP, dTTP, dCTP and dGTP), 300 nM of each primer, 1x PCR buffer and 2.6 units of Expand Long Template PCR system, a mixture of Taq and Tgo DNA polymerases (Barnes, 1994; Cheng *et al.*, 1994). Samples were overlaid with 30 µl of mineral oil and PCR was performed in a Perkin Elmer Cetus DNA thermal cycler. PCR was usually carried out for 30 cycles (denaturation: 95°C for 30 sec per cycle; annealing: 50°C for *pknA* or 55°C for *ppp* for 30 sec per cycle; elongation: 68°C for 2 min for first 10 cycles and then for the remaining 20 cycles the elongation step was extended for an additional 20 sec in each cycle). ‘CAT’ was introduced in the 5’ end primers to incorporate a *Ndel* site in the PCR amplified fragment (Tables 2.2 and 2.3). PCR markers or DNA
ladders were run along with PCR products on agarose gels (0.8% - 1.2%) for appropriate size analysis of the amplified fragments.

2.2.11 DNA sequencing and analysis

Sequencing grade DNA was prepared using ABI (miniprep) kit as described in Section 2.2.1. Both commercially available as well as custom synthesized primers were used in PCR reactions. PCR was carried out for 24 cycles (denaturation: 96°C for 30 sec per cycle; annealing: 50°C for 15 sec per cycle and elongation: 60°C for 4 min per cycle). The PCR samples were further processed for sequencing. DNA was precipitated with ethanol and sodium acetate (pH 4.6). The pellet was washed with 70% ethanol and air-dried. DNA was denatured following the addition of template suppression reagent (TSR). Sequencing was carried out on an automated sequencer (ABI PRISM 377 DNA Sequencer, Perkin Elmer Applied Biosystems).

2.2.12 Southern/Northern blotting and hybridization

Blotting: For Southern transfer of DNA fragments, the capillary method as described by Sambrook et al. (1989); Sambrook and Russel (2001) was followed. Briefly, digested genomic DNA (3 - 5 µg), PCR reaction products or digested plasmid constructs were resolved by electrophoresis. The gels were treated sequentially for 60 min each in the denaturation (1.5 M NaCl in 0.5 M NaOH) and neutralization (1.5 M NaCl in 0.5 M Tris, Cl pH 7.4) solutions. DNA from the gel was transferred to nylon membrane in 6x SSC. After 20 hours of transfer, the membrane was washed with 6x SSC, air-dried and DNA was fixed by UV cross-linking at 0.15 Joules per sq. cm (Vilber Lourmat Fluo-Link, France). These membranes were used for hybridization. For Northern blotting, formaldehyde-agarose denaturing gels were washed with DEPC treated water and capillary transfer was set up. Further processing of the membrane was carried out in a similar manner as described for Southern blotting.

Preparation of probes: Random priming or End labelling methods described by Davis et al. (1994) were followed for ³²P labelling of DNA fragments and oligonucleotides to be used as probes for Southern/Northern/oligo hybridization.
a) **Random priming:** DNA fragments were labelled internally by random priming. 25 – 50 ng of DNA template was mixed with 40 ng random hexamers in a final volume of 6 µl. Sample was denatured in a boiling water bath for 5 min and then rapidly chilled in an ice-water bath. 14 µl of labelling mix containing 1x Klenow buffer, 20 µM GAT mix (dGTP, dATP and dTTP), 0.66 µM \(\alpha\text{-}^{32}\text{P}\)-CTP and 5 units of Klenow enzyme was added to the denatured sample. The reaction was incubated at room temperature for 45 min and stopped by adding 80 µl TE containing 1 µg ssDNA (carrier DNA). The reaction mixture was precipitated with two volumes of ethanol at -70°C for 2 h. The labelled probe was pelleted at 10,000xg for 15 min, washed with 70% ethanol and finally dissolved in TE.

b) **End labelling:** Oligonucleotides were labelled with \(\gamma\text{-}^{32}\text{P}\)-ATP using T4 polynucleotide kinase. The reactions were incubated at 37°C for 1 h and stopped by the addition of TE. The contents were filtered through Costar Spin-X 0.22 µM columns. The filtrate containing the labelled probe was collected.

An aliquot of labelled DNA was taken and radioactive counts were determined by Cerenkov counting in a liquid scintillation counter.

**Hybridization:** The membranes carrying blotted DNA/RNA were prehybridized with 100 µg/ml sheared ssDNA in 6x SSC, 0.5% SDS, 5x Denhardt solution and 50% formamide for 2 - 4 h at 42°C. Blots were hybridized with \(^{32}\text{P}\) labelled probes (denatured by boiling for 10 min) at 42°C in the same buffer for 12 - 16 h. This was followed by successive washings with 2x SSC containing 0.5% SDS for 5 min at room temperature, 2x SSC with 0.1% SDS for 15 min at room temperature, 0.1x SSC with 0.5% SDS at 37°C for 1 h and 0.1x SSC with 0.5% SDS at 65°C for 1 h. For less stringent conditions, prehybridization and hybridization were carried out at 37°C in the buffer containing 40% formamide. When \(^{32}\text{P}\) labelled oligonucleotides were used as probes, hybridizations were carried out in 6x SSC, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA, 0.5% SDS, 0.1% non-fat dried milk and 100 µg/ml sheared ssDNA. After hybridization, the membranes were washed with 6x SSC and 0.1% SDS for 5 min at room temperature, followed by 15 min each at 37°C and at the
temperature of hybridization. Final washing was done with 2x SSC and 0.5% SDS at a temperature 5°C lower than the temperature of hybridization. The blots were exposed to X-ray films and autoradiographs were developed.

Membrane stripping: The probes were stripped from nylon membranes by incubating these at 45°C for 30 min in 0.4 N NaOH followed by neutralization for 15 min at the same temperature in 0.1x SSC, 0.1% SDS and 0.2 M Tris Cl (pH 7.5). The removal of the probe was confirmed by autoradiography of stripped blots.

2.2.13 Site-directed mutagenesis

PCR was employed to generate point mutants of \textit{M. tuberculosis} pknA and \textit{ppp}. Two external and two internal primers were synthesized for each mutation. Base mismatches for desired mutations were incorporated in the internal primers. To generate a mutant, two sets of primary and one set of secondary PCR reactions were carried out by overlap extension method (Ho et al., 1989) as illustrated in Fig. 2.1. Primary reactions were carried out with one external and one internal primers using wildtype construct as the template. Finally, mixture of primary PCR products for individual mutant served as template for carrying out secondary reactions with the external primers. PCR conditions were same as used for wildtype gene amplification (Section 2.2.10). Details of specific mutations are provided in Chapters 4 and 5.

2.2.14 Protein estimation

Protein was estimated according to the method of Bradford (1976) using BSA as the standard. 500 µl of the samples (diluted when necessary) were mixed with 500 µl of Bradford reagent (commercially available from Sigma) by vortexing and absorbance was monitored at 595 nm in a Beckman spectrophotometer (DU 640B).

2.2.15 SDS-PAGE

SDS-PAGE was carried out essentially according to Laemmli (1970). Briefly, protein samples were prepared by mixing with 5x sample buffer at a final concentration of 1x. The samples were boiled for 5 min and centrifuged at 10,000xg.
Fig. 2.1 Schematic diagram of site-directed mutagenesis by overlap extension. The double stranded DNA and primers are represented by lines with arrows indicating the 5' to 3' direction. The site of mutations is indicated by small cross. PCR products are denoted by pairs of upper-case letters corresponding to the primers used to generate that product. The boxed portion of the figure represents the intermediate steps where the denatured fragments anneal at the overlap and are extended 3' by the Expand Long Template PCR system. A / D and B / C indicate external and internal primers respectively. EcoRI and NdeI represent restriction enzyme sites incorporated in the external primer A.
prior to loading onto the gel. The discontinuous gel system had 8 - 12.5% resolving gel (depending on the size of the protein) in 0.375 M Tris. Cl, pH 8.8 and a 5% stacking gel in 0.125 M Tris. Cl, pH 6.8. Gels were run in Laemmli buffer at a constant current of 20 mA till samples entered the resolving gel and then at 30 mA upto the completion of gel run. Protein molecular weight markers were also run concurrently on the gels. The gels were stained with 0.1% Coomassie brilliant blue R-250 in 10% glacial acetic acid and 40% methanol for ~1 h and then destained in 10% glacial acetic acid and 40% methanol.

2.2.16 Expression and purification of recombinant proteins

pknA and ppp genes or their mutants cloned in pMAL-c2 were transformed in E. coli strain TB1 and selected on LB ampicillin plates (100 µg/ml). Overnight cultures were reinoculated and grown to an OD_{600} of 0.5. Cells were induced with 0.3 mM IPTG for 3 h. pknA cloned in pGEX-KG was transformed in E. coli strain DH5α and the reinoculated culture was induced with 0.2 mM IPTG at an OD_{600} of 1.0. In order to check the expression of recombinant proteins, cells were harvested by centrifugation, washed and finally suspended in lysis buffer. The cell suspension was boiled for 5 min following the addition of sample buffer. The lysate was centrifuged at 9,600xg for 15 min and loaded on SDS-PAGE. To know the solubility of the expressed fusion proteins, cells after resuspension in lysis buffer were sonicated. Supernatant and pellet fractions obtained were subjected to SDS-PAGE. In order to examine the constitutive expression of protein and its solubility, overnight cultures (at 37°C) of constructs in p19Kpro were processed similarly as mentioned for constructs cloned in pMAL except that IPTG induction was not required. The GST and MBP fusion proteins were purified by affinity chromatography on glutathione agarose (Sigma) and amylose columns (New England Biolabs) respectively. Details of purification are provided in Chapters 3 and 5. The MBP fusion proteins of PknA and PPP were designated as MBP-PknA and MBP-PPP respectively.
2.2.17 Western blotting

Purified fusion proteins or cell extracts (800 ng - 3 µg protein/slot) were resolved in 8 or 10% SDS-PAGE and transferred at 250 mA for 45 min to nitrocellulose membrane (0.45 µm) in a mini-transblot apparatus (Bio-Rad) using Tris-Glycine-SDS buffer (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol, pH ~8.3). After electroblotting, nitrocellulose membranes were stained with Ponceau S to verify protein transfer. Membranes were washed with TBS and blocked for 1 h at room temperature or overnight at 4°C in TBST containing 5% skimmed milk or BSA. These were further incubated either overnight at 4°C or 2 h at room temperature with primary antibody diluted in the blocking solution. The membranes were washed four times with TBST for 15 min each and then incubated with anti-mouse or anti-rabbit HRP conjugated secondary antibody for 1 h at room temperature. After thorough washing with TBST (four times for 15 min each), the blots were processed using ECL Plus Western blotting detection kit (Amersham Pharmacia), which is based on the HRP and peroxide catalyzed oxidation of the Lumigen PS-3 acridan substrate. The acridinium ester intermediates generated by the oxidation of acridan react with peroxide under slight alkaline conditions to produce a sustained, high intensity chemiluminescence.

2.2.18 ELISA

The titer of polyclonal antibody generated in rabbit was checked by indirect enzyme linked immunosorbent assay (ELISA). The fusion protein, MBP-PknA, suspended in carbonate-bicarbonate buffer was coated overnight at 4°C on an ELISA plate. The plate was washed with PBS, pH 7.4 to remove unbound antigen and blocked with 10% skimmed milk in PBS for 2 h at 37°C. After three washes with PBST and one with PBS, further incubation was carried out with primary antibody for 2 h at 37°C. The plate was washed again with PBST thrice and once with PBS and incubated with anti-rabbit HRP conjugated secondary antibody (1:5000) at 37°C for 1 h. The antibodies were diluted in PBS containing 0.1% skimmed milk. Following thorough washing with PBST and PBS, the plates were incubated with substrate
solution 3, 3', 5, 5'-Tetramethylbenzidine (TMB) for 30 min at 37°C. The reaction was stopped by the addition of 1 N H₂SO₄. Absorbance of the coloured product was measured at 450 nm in an ELISA reader (Emax Molecular Devices).

2.2.19 Kinase and dephosphorylation assays

Ability of PknA or its mutants as purified fusion protein with MBP for autophosphorylation and also for phosphorylating exogenous substrates like histone (from calf thymus, type II-AS, Sigma), myelin basic protein (from bovine brain, Sigma) or casein (from bovine milk, Sigma), was determined in an in vitro kinase assay. Aliquots (usually 800 ng to 6 µg/20 µl reaction volume) of fusion protein were mixed with 1x kinase buffer (50 mM Tris Cl pH 7.5, 50 mM NaCl, 10 mM MnCl₂) and the reaction was initiated by adding 2 µCi of [γ⁻³²P]-ATP. Following incubation at room temperature for 20 min, the reaction was stopped by adding SDS sample buffer (30 mM Tris Cl pH 6.8, 5% glycerol, 2.5% β-mercaptoethanol, 1% SDS and .01% bromophenol blue). Samples were boiled for 5 min and resolved on 8 - 12.5% SDS-PAGE. Gels were stained with Coomassie brilliant blue, dried in a gel dryer (Bio-Rad) at 70°C for 2 h and analyzed in a phosphoimager (Molecular Imager FX, Bio-Rad) using ‘Quantity one’ software and also exposed to Kodak X-Omat/AR film. Autophosphorylating ability of the constitutively expressed PknA was determined in a similar manner.

For dephosphorylation studies, MBP-PPP was added to the reaction mix containing γ⁻³²P labelled protein and incubated at 37°C for different time periods. The reaction was terminated by the addition of sample buffer and subjected to SDS-PAGE. Gels were dried and analyzed as described above.

2.2.20 In vitro interaction studies with E. coli lysate

MBP-PknA (100 µg) was immobilized on amylose resin and incubated in the presence of soluble protein extracts (250 µg) prepared from E. coli strain DH5α for 10 h at 4°C. Amylose beads were washed (4,500xg for 5 min) four times to remove unbound proteins. After suspension of washed beads in TEN buffer, aliquots were
labelled with $\gamma^{32}\text{P}$ at room temperature for 30 min. Dephosphorylation was then initiated by the addition of 2 $\mu$g MBP-PPP. The reactions were incubated at 37°C, terminated after 1 h with SDS sample buffer and processed as described in Section 2.2.19.

2.2.21 Phosphatase assay

The phosphatase activity of PPP was assayed using pNPP as substrate following the method of Zhuo et al. (1993) with slight modifications. The assay was performed in microtiter plate in a total reaction volume of 50 $\mu$l. The reaction was carried out in 50 mM Tris. Cl, pH 8.0 containing 3.5 mM pNPP, 5 mM MnCl$_2$ and 8 $\mu$g of the purified fusion protein at 37°C for 15 min. Increase of p-nitrophenol was monitored at 405 nm in an ELISA reader. Specific activity of phosphatase is expressed as nanomoles of p-nitrophenol liberated /min/mg of protein.

2.2.22 Scanning electron microscopy

Overnight cultures of E. coli strain DH5$\alpha$ alone or transformed with different constructs were reinoculated such that initial OD$_{600}$ was 0.05 and were grown further for 12 h. Following harvesting, cells were washed three times with ice cold PBS. The cells were then resuspended in PBS, adhered to coverslips that had been coated with 0.1% poly L - lysine. Adherent cells were washed with PBS and then dehydrated using an ascending series of ethanol (30% - 100%) incubations (each step of 30 min). Finally, cells on cover slips were infiltrated with ter-butyl alcohol and freeze dried in a lyophilizer (Inoue and Osatake, 1988). Dried samples were sputter-coated with gold/palladium and then observed under a scanning electron microscope.

2.2.23 Bioinformatic analysis

Nucleotide derived amino acid sequences were compared with non-redundant database in PSI-BLAST or BLASTP programme using mail server at NIH (Altschul et al., 1990; 1997). The multiple sequence alignments of the retrieved sequences were carried out using the Clustal W 1.74 (Thompson et al., 1994) or Clustal X
(Thompson *et al.*, 1997) programme. The gap opening and extension penalties of 10 and 0.05 respectively were used during the alignments. The conserved regions were shown by shading the sequence using BioEdit programme (Hall, 1999). During shading the standard colour scheme for each amino acid was followed. The multiple sequence alignments for generating the phylogenetic tree were performed by excluding highly variable amino- and carboxy- terminal stretches of the sequences. The trees were finally constructed by neighbor-joining method (Saitou and Nei, 1987) or PROTDIST, UPGMA and CONSENSE programmes available at the PHYLIP site (Felsenstein, 1993) and drawn with TREEVIEW (Page, 1996).

### 2.2.24 Data analysis

All experiments were done at least three times to ensure the reproducibility of data. The data was expressed as Mean ± SD. Statistical analyses have been carried out by Student’s t-test and p<0.05 has been considered as minimum level of significance.