CHAPTER 2

MATERIALS AND METHODS
2.1. In silico analyses of PPE family ORFs

*In silico* analysis of a few of the PPE ORFs belonging to the third subgroup of the PPE family was carried out using the protein analysis software (Protean 4.0, Lasergene Navigator, DNASTAR Inc., Madison, WI) namely Kyte-Doolittle Hydrophilicity plot, Jameson-Wolf antigenic index and the Emini surface probability plot.

2.2. RNA Extraction

RNA was extracted from liquid cultures of H37Rv. The strain was grown in MiddleBrooks 7H9 liquid broth (Difco, USA). RNA was extracted using the Qiaquick Total RNA extraction kit (Qiagen, USA). The manufacturer’s protocol was modified to get better yields of RNA from mycobacteria. All the reagents were diethyl pyrocarbonate (DEPC) treated to inhibit RNAse activity. A loop full of cells (~$10^9$) was transferred from the liquid media to 1 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The cells were centrifuged at 10,000 rpm for 10 minutes to collect the cell pellet. The soup was discarded and 100 µl of fresh TE containing 10 mg/ml lysozyme was added and the tube incubated for 15 minutes at 37°C. The cells were lysed by adding 350 µl buffer RLT (containing 4 M guanidine iso-thiocynate, 0.25 M sodium citrate, 0.1% sarcosine, 0.2 M sodium acetate, 0.7% (3-mercaptopethanol). The sample was vortexed vigorously and incubated at 65°C for 1 hour. The cell debris was centrifuged for 2
minutes at 12,000 rpm in an eppendorf rotor using a table top Heraeus centrifuge. The supernatant was transferred to a fresh tube. 250 μl of absolute ethanol was added and mixed by inverting the tube a couple of times. This was then put into the spin column (containing the silica matrix). The tube was centrifuged at 8,000 rpm for 1 minute. The flow through was discarded and 700 μl of buffer RW1 (as per the instructions given in the RNeasy Mini Handbook, Qiagen, USA) was added. The tube was centrifuged at 8,000 rpm for 15 seconds, the flow through was discarded and 500 μl of buffer RPE (containing ethanol for wash) was added. The tube was centrifuged at 8,000 rpm for 15 seconds. This step was repeated to wash out all the impurities. A final centrifugation at 10,000 rpm for 1 minute ensured that the silica matrix was dry and no carry over of Buffer RPE took place. The tube was transferred to a clean 1.5 ml tube and 50 μl of nuclease free water was added into the tube. The tube was centrifuged at 8,000 rpm for a minute to collect the dissolved RNA and stored at -70°C till further use.

2.3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Primers designed on the basis of the published sequence of Rv2430c were used for the RT-PCR reaction. Promega Access RT-PCR kit was used for the amplification of RNA. A 50 μl reaction contained ~100 ng
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of RNA, 1 μM each of the oligonucleotide primers specific to the corresponding gene, 0.2 mM each of the dNTPs, 1X of the provided reaction buffer, 1mM of MgSO₄ and 0.1 U/μl each of AMV Reverse Transcriptase and Tfi DNA Polymerase. For the first strand synthesis the tube was incubated at 48°C for 45 minutes followed by incubation at 96°C for 2 minutes to inactivate the reverse transcriptase. The second strand synthesis and PCR was performed using the following conditions; 25 cycles of 94°C-1 minute, 48°C-1 minute, and 68°C-2 minute. One final hold at 68°C for 10 minutes ensured the synthesis of complete length amplicons. In the control series two reactions were set up. One had no reverse transcriptase to check for DNA contamination. The second had no template to check for aerosol contamination. The other reaction conditions were identical to sample RT-PCR conditions. All other routine precautions of handling RNA and setting up of PCR were observed to limit chances of contamination. The PCR products were resolved by electrophoresis on 2% agarose gel.

2.4. Genomic DNA Extraction from H37Rv

Conventional Protocol

The *M. tuberculosis* cells (10⁷ cells) were scraped from Lowenstein-Jensen agar slants and suspended in 1 ml of TE (10 mM TrisCl, 1
mM EDTA pH 8.0). The cells were killed by three cycles of boiling at 100°C followed by freezing at -70°C. The cells were pelleted by centrifugation at 12,000 rpm for 10 minutes. The pellet was resuspended in 500 µl of fresh TE. Dead cells were lysed by incubation at 37°C with 100 µg/ml lysozyme for 30 minutes. The cells were vortexed for one minute and 2% Sodium dodecyl sulphate (SDS) and 15 fg/ml Proteinase K were added and the tubes were incubated at 65°C for 2 hour. The proteins, carbohydrates and other contaminants were selectively precipitated out with 10% Cetyl trimethyl ammonium bromide (CTAB) and 0.7M NaCl. After addition of CTAB-NaCl the tubes were incubated at 65°C for 15 minutes. The tubes were then cooled on ice for 10 minutes. This was followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected in a fresh tube and equal amount of phenol chloroform isoamyl alcohol (25:24:1) was added. The centrifugation step was repeated and the aqueous phase was collected in a fresh tube. To precipitate the DNA, 0.7 volumes of isopropanol was added and mixed by inverting the tubes a couple of times. The tubes were kept at 4°C for 2 hours. The DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was washed with 70% ethanol to remove salt. The DNA was finally resuspended in TE (10 mM TrisCl, 1 mM EDTA pH 8.0).
2.5. Polymerase Chain Reaction (PCR) amplification of Rv2430c

The Rv2430c gene was PCR amplified from the genomic DNA of H₃₇RV using upstream (5' -GGATCCATGCATTTCGAAGCGTAC- 3') and downstream (5' -AAGCTTCTAAGTGTCTGTACGCGATGA- 3') primers. BamHI and HindIII sites were incorporated in the 5' and 3' of the primers respectively, to facilitate directional cloning. For amplification, the Taq DNA polymerase (Promega, USA) was used. The PCR was set up in a 50 µl volume containing 100 ng of template (genomic DNA from the isolate), 1X Reaction buffer, 0.2 mM each of dNTPs, 1 unit of Taq DNA polymerase, 1.5 mM MgCl₂, 100 ng each of the primers and distilled water to make up the volume. The template was added to initiate the reaction and the tubes transferred to the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, USA). The amplification reaction was carried out as per the following conditions:

Denaturation 94°C - 2 min.
Annealing 48°C - 90 sec.
Extension 72°C - 2 min.

These conditions were repeated for 30 cycles and the resulting PCR product (amplicon) was subsequently used for cloning purposes.
2.6. DNA Purification

Once the amplicon was checked on the agarose gel, it was purified either by Qiagen’s Qiaquick PCR purification kit or Qiaquick gel extraction kit. The former was used in cases where there was no non-specific amplification. In cases where there were more than one bands the gel extraction protocol was applied. These kits employ the technique of binding DNA to a silica matrix in the presence of high salt buffers. While the DNA stays bound to the matrix, the impurities are washed out by 70% ethanol. This DNA can then be eluted by TE or water. The DNA so obtained is of high quality with the O.D. A260/A280 in the range of 2.0.

2.7. PCR purification

The PCR product was diluted in 5 volumes of Buffer PB. The spin column was placed in a 2 ml collection tube and the diluted mix added to the column. The Buffer PB provides the high salt conditions for DNA binding to the silica matrix in the column. The spin column was centrifuged at 8,000 rpm for a minute. The flow through was discarded and the column was put back in the collection tube. The bound DNA was given two washes with 0.5 ml Buffer PE (ethanol containing buffer). To completely dry the tube to make it free of any
solutions a final spin for 2 minutes was given and the spin column was transferred to a fresh 1.5 ml tube. The DNA was eluted by adding 50 µl of distilled water to the column and the tube centrifuged at 8000 rpm for a minute. The DNA was checked for quantity and quality by running an aliquot (5 µl) on a 2% agarose gel. The tube was stored at -20°C till further use. It was important to dissolve the DNA in water and not in TE as the EDTA in the buffer inhibits the PCR sequencing step. All the samples that needed to be sequenced were dissolved in distilled water.

2.8. Gel Extraction

For gel extraction the remaining (usually 40 µl) sample was loaded on 2% agarose gel and electrophoresed in 1X TAE buffer. The DNA was resolved and the specific band (based on the amplicon's molecular weight) was excised. DNA was extracted from the gel slices using the QIAquick Gel Extraction kit (Qiagen, USA). The gel slice was weighed and 3 volumes of Buffer QG (for solubilizing the gel piece and for providing the right condition for DNA binding to the silica matrix in the spin column) were added to it. The tube was incubated at 55°C for 15 minutes with gentle agitation. 1 volume of isopropanol was added to the mix and vortexed. This was then transferred to the spin column placed in a 2 ml collection tube. To bind the DNA the column
was given a brief spin at 8,000 rpm for a minute. The flow through was discarded and 0.5 ml of Buffer PE (ethanol containing buffer) was added to the column. The column was placed in the collection tube and centrifuged as above. This wash step was repeated once more to remove unwanted primers, salts, enzymes, unincorporated nucleotides etc. The spin column was then given a dry run that is without any buffers. This ensured that the column was free of any solutions and the resin within the column was completely dry. The spin column was transferred to a fresh 1.5 ml tube and 50 µl of distilled water was added. The tube was spun at 8,000 rpm for a minute to elute the DNA. The tube was transferred to -20°C after an aliquot (5 µl) of the eluate was electrophoresed on an agarose gel to confirm quantity and quality of DNA.

2.9. Cloning of the PPE gene Rv2430c

The purified fragment was ligated into the pGEMT-easy vector (Promega Inc., USA) and the ligation mixture was then transformed into competent DH5α cells using standard protocols. The transformed cells were plated on LB agar with 100 µg/ml ampicillin and the resulting colonies were screened for the insert. The insert was then sequenced from the vector using T7 promoter primer.
2.10. **Automated DNA Sequencing**

Sanger's Dideoxy method was used for sequencing the DNA samples. The technique depends on the requirement of the growing chain of single strand DNA for a 3' hydroxyl group to form a phosphodiester bridge with the incoming nucleotide. DNA polymerases are capable of incorporating analogues of nucleotide bases. So instead of a 2', 3' deoxynucleotide (dNTP) 2', 3' dideoxynucleotide (ddNTP) is added which does not have the hydroxyl group needed to form the phosphodiester bond with the incoming nucleotide. This leads to chain termination at this point. In automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension product using 3'-dye labeled dideoxy nucleotide triphosphates (ddNTP). Each of the four different ddNTP (A, G, C, T) is labeled with a different dye that emits fluorescence at different wavelength. So the ddNTP that terminates the chain can be determined by the colour it emits. This varying positional colour-code is read on the automated sequencer and the DNA sequence obtained. Cycle sequencing is a simple method in which successive rounds of denaturation, annealing, and extension in a thermal cycler result in linear amplification of the extension products. The advantages of the technique are- the sequencing reaction can be done in a single tube, there is requirement of less DNA template and more samples can be
sequenced per gel (instead of four well per sample in radiolabeled DNA sequencing, here one sample is loaded in one well of the gel). The sequence lengths of 600 bases can be read with an accuracy of 95%. Both strands were sequenced to ensure the accuracy of sequencing.

2.11. Cycle Sequencing Reaction

For each sample two sequencing reactions, using forward or reverse primer were set up. The sequencing primers were identical to the ones used in PCR amplification of the different loci. PCR sequencing was carried out using the BigDye terminator kit (ABI Prism, USA) according to the manufacturer's instructions. Each sequencing reaction of 20 μl volume consisted of 50 ng template, 5 pmoles primer, 8 μl sequencing mix (AmpliTaq DNA polymerase, FS, with thermally stable pyrophosphate, the 4 dNTPs, MgCl2, Tris-HCl buffer pH 9.0, 4 ddNTPs labeled with 4 different dyes) and distilled water to 20 μl. The cycling parameters were as follows; 30 cycles of 96°C for 30 seconds; 45°C-60°C for 30 seconds (depending on the Tm of primer used); 60°C for 3 minutes. The labeled extension product was purified to remove unincorporated labeled ddNTPs. This was done by adding 0.1 volumes of 3 M sodium acetate pH 4.5 and 2.5 volumes of absolute ethanol. The solutions were mixed by vortexing and the
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tube was left at room temperature for 10 minutes. The tube was centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The soup was discarded and the pellet was washed twice with 70% ethanol. After the final wash the tubes were air-dried. The pellet was dissolved in 5 µl of loading dye (90% Formamide, containing 0.1% blue dextran) and heated at 95°C for 3 minutes to denature the DNA. The tube was kept on ice till loading and the left over was stored at-20°C. 1.5-2.0 µl of the sample was loaded onto the 5% polyacrylamide gel (19:1 acrylamide-bis acrylamide, 1X TBE buffer) and electrophoresed at 3000 Volts for 3 hours in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). Sequencing of the amplicons was carried out using the ABI Prism Automated DNA Sequencer 377 sequencer (ABI Prism, USA). The automated sequencer was set to collect data with filter set E as recommended by the manufacturer. The software package Sequencing Analysis 3.3™ was used for analyzing the gel information. Sequences generated by the program were compared with their respective wild type sequences using MegAlign software (Lasergene, DNASTAR, Inc USA).

2.12. Subcloning of Rv2430c in PQE30 expression vector

After confirmation of the sequence the insert was extracted from the pGEMT-easy vector by digestion with BamHI and HindIII enzymes
and then subcloned into the pQE30 expression vector (Qiagen Inc., USA), downstream of a 6X histidine sequence tag, at the *Bam*HI and *Hind*III sites to generate the plasmid construct called PQERv2430c. The ligation mix contained 100ng each of the vector and the insert, 1U DNA ligase, 1X reaction buffer and the volume was made up with water. The reaction was allowed to proceed at 14°C for 12 hours and the ligation mix was transformed in DH5α cells using the standard protocols. The resulting construct PQERv2430c was purified and was finally transformed into M15pREP4 strain of *E.coli* for expression of the recombinant protein.

### 2.13. Expression and purification of the recombinant protein coded by Rv2430c

A single colony of M15pREP4 cell line harbouring the construct was inoculated in 5 ml of LB broth with the appropriate antibiotics, as mentioned above, and grown overnight at 37°C with constant agitation. 100μl of this overnight culture was inoculated into 5 ml of LB broth with the appropriate antibiotics and grown till a cell density corresponding to an absorbance value of 0.6 (A<sub>590</sub> = 0.6) was achieved. The culture was then induced with 1 mM IPTG. A separate aliquot of uninduced culture was kept as a control. Cells were harvested 3 hours post induction, suspended in 1X SDS sample buffer and
denatured by heating at 100°C for 10 min. The samples were resolved in a 12% SDS polyacrylamide gel, confirming that the expected 23 kDa protein was expressed.

The recombinant protein was then purified to homogeneity using the QIAExpressionist kit (Qiagen, Inc., USA). Cells harvested from 10 ml of induced culture were resuspended in lysis buffer containing 100 mM NaH$_2$PO$_4$, 10 mM Tris.Cl and 8M urea (pH 8.0). The lysate was loaded onto a Ni-NTA column pre-equilibrated with the lysis buffer. The column was washed with wash buffer containing 100 mM NaH$_2$PO$_4$, 10 mM Tris.Cl and 8 M urea (pH 6.3). Finally, the protein was eluted with elution buffer containing 100 mM NaH$_2$PO$_4$, 10 mM Tris.Cl and 8 M urea (pH 4.5), and resolved by electrophoresis in a 12% SDS polyacrylamide gel. A single 23 kDa protein band was observed upon staining with Coomassie Brilliant Blue dye, indicating the purified protein. The protein was then dialyzed against 1 X PBS, pH 7.5 using a 10 kDa cut-off dialysis membrane and the dialyzed protein was quantified using the PIERCE Micro BCA Protein Assay Reagent Kit, according to the manufacturer's instructions.

### 2.14. Human Study Population

Serum samples were obtained from 101 TB patients who reported to the outpatient department of Mahavir Hospital and Research Centre,
Hyderabad. These 101 patients belonged to four well classified categories of patients. Category 1 (n=32) comprised patients who had contracted TB for the first time and had no history of TB treatment. Category 2 (n=30) comprised patients with relapsed TB, i.e., who were treated earlier for TB but the symptoms resurfaced after the completion of treatment. Category 3 (n=32) comprised patients with extrapulmonary TB in which case the disease was confirmed by tissue biopsy and category 4 (n=9) were patients with MDR TB. In the case of category 1 and category 2 patients, diagnosis was confirmed by the examination of the sputum (acid-fast bacilli smear positive). 10 clinically healthy donors, which were *M. bovis* BCG vaccinated, were included in the study. 15 non TB patients, i.e patients which harbored any pathogen but were culture negative for *Mycobacterium tuberculosis* were also included in the study. The study was carried out after approval from the Institute Bioethics Committee.

2.15. Serological characterization of the recombinant protein

Serological characterization of the recombinant protein was carried out using ELISA (Enzyme linked immunosorbent assay). ELISAs were performed in 96-well microtitre plates (Corning, Costar) coated with the recombinant Rv2430c protein. After overnight incubation at 4°C, the plates were washed three times with phosphate-buffered saline
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(PBS) buffer and blocked with 200μl of blocking buffer (PBS containing 1% bovine serum albumin) for 1h at 37°C. The plates were then washed with PBS-Tween wash buffer (0.05% Tween 20 in 1X PBS, pH 8.0) and incubated for 1h at 37°C with human sera (1:200 dilution in blocking buffer). The plates were washed with PBS-Tween and further incubated with either anti-human immunoglobulin G (IgG)-horseradish peroxidase (HRP) or anti-human IgM-HRP (Sigma). HRP activity was detected using a chromogenic substance, o-phenylenediamine tetrahydrochloride (Sigma) in citrate-phosphate buffer (pH 5.4) and 1μ of H₂O₂/ml. The reactions were terminated using 1 N H₂SO₄ and the absorbance values were measured at 492nm in an ELISA reader (Bio-Rad, USA).

2.16. Localization of Rv2430c using an in-vitro coupled transcription and translation system

Localization of Rv2430c was checked by using a coupled transcription-translation system. The ORF Rv2430c was cloned in pET23a expression vector under the control of T7 promoter to generate a construct pET23aRv2430c. The coupled transcription and translation reaction was carried out using the TNT Coupled Reticulocyte Lysate Systems as per the suppliers instructions (Promega, USA). Briefly, 1μg of the plasmid (pET23aRv2430c), 0.5μl of
amino acid mix, 1μl of RNAsin, 1μl of \(^{35}\)S Methionine, 0.5μl of T7 RNA polymerase, 13μl of Rabbit Reticulocyte Lysate, 1μl of reaction buffer and 6μl of water was incubated at 30°C for 90 minutes. A separate reaction with the above reaction mix plus Canine Pancreatic Microsomal membrane was also set up. Both the reaction products were treated with Trypsin and analysed on SDS-PAGE.

2.17. Statistical analysis

Student's t-test was used for analysis of statistical significance (p-value). Graphpad Quickcalcs (Online t-test calculator) was used for this purpose. http://www.graphpad.com/quickcalcs/ttest1.cfm.

2.18. Secondary structure prediction of Rv2430c

*In silico* analysis of the Rv2430c was carried out using the Protein analysis software (Protean 4.0, Lasergene Navigator, DNASTAR Inc., Madison, WI). Predict protein (http://cubic.bioc.columbia.edu/predictprotein/) and PSIPRED (http://bioinf.cs.ucl.ac.uk/psiform.html) both of which were web based.
2.19. On-column refolding of recombinant Rv2430c

A 100ml culture of M15pREP4 strain carrying the recombinant plasmid PQERv2430c was grown till log phase and induced with 1mM IPTG. Cells were harvested 3 hours post induction and suspended in Buffer A (25mM Tris-Cl, pH 8.0 containing 8M urea and 0.9% NaCl) and incubated on an end-to-end shaker for 30 minutes at room temperature for lysis. The lysate was centrifuged at 13000 rpm for 30 minutes and the supernatant was then incubated with pre-equilibrated Ni-NTA slurry (Qiagen Inc., USA) for 15-20 minutes with gentle agitation to maximize the binding of the recombinant protein. The protein bound to slurry was then packed into a column. The bound protein was then subjected to on-column refolding by using a 250 ml gradient of buffer A and Buffer B (25mM Tris, pH 8.0, 5mM imidazole, 1mM glutathione and 0.1M L-Arginine hydrochloride) at a flow rate of 1ml/min using Acta-Prime chromatographic unit (Pharmacia Biotech). At the end of the gradient, the column was further washed with 50 ml of buffer B and then eluted with 25mM Tris, pH 8.0 containing 500 mM imidazole. The homogeneity of the eluted protein was confirmed by 12% SDS-PAGE and the purified protein was dialyzed extensively at 4°C against 25mM Tris HCl pH 8.0 containing 0.9% NaCl. Protein was quantified by Pierce Micro BCA Protein Assay Reagent kit (Pierce,
USA) and was subsequently used for spectroscopic analyses. The dialyzed protein was further centrifuged at 13000 rpm for 15 minutes to check for the presence of any visible aggregation.

2.20. Circular Dichroism (CD) Spectroscopy

CD measurements were carried out on a spectropolarimeter (JASCO-715, JAPAN) using a 0.02-cm cell at 0.2-nm intervals and a two-nanometer bandwidth. Spectra were signal averaged by adding at least 4 accumulations. The base line was corrected by subtracting the spectra of respective buffer blank obtained under identical conditions. Percentage of secondary structure was calculated using the web based programme K2D (http://www.embl-heidelberg.de/~andrade/k2d/).

2.21. Fluorescence spectroscopy

Purified recombinant Rv2430c protein was incubated in the presence or absence of 8M urea for 2h at room temperature and the fluorescence emission spectra (300-400nm) were recorded by exciting the protein at 280nm using Perkin-Elmer LS-3B spectrofluorimeter. The slit width was 10nm and the scan speed was 50nm/sec.