MATERIAL AND METHODS
MATERIAL AND METHODS

3.1 Materials

3.1.1 Reagents/Chemicals
All the chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Hi-Media (India). The growth media for culturing purpose was purchased from Hi-Media (India). Tissue culture medium, DMEM (Dulbecco's Modified Eagle’s Medium), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), Lipopolysaccharide (LPS) of Escherichia coli 0111:B4, sulfanilamide, naphthylethylene-diamine dihydrochloride and phosphoric acid (H₃PO₄) were purchased from Sigma Chemical Co., St. Louis, Mo. Fetal calf serum (FCS) and fetal bovine serum (FBS) were procured from Biowest (France).

3.1.2 Substrates/inhibitors
All the substrate that include para-nitrophenyl (pNP)-acetate (C₃), pNP-butyrate (C₄), pNP-deconate (C₁₀), pNP-palmitate (C₁₂), pNP-myristate (C₁₄), pNP-palmitate (C₁₆), pNP-stearate (C₁₈) and inhibitors like phenylmethylsulfonyl fluoride (PMSF), eserine, Diethyl pyrocarbonate (DEPC) used were purchased from Sigma-Aldrich Chemicals Co. (USA).

3.1.3 Antibiotics/IPTG/X-gal
Ampicillin, kanamycin, IPTG and X-gal were purchased from Hi-Media (India) and streptomycin and penicillin were purchased from Sigma Chemical Co., St. Louis, Mo.

3.1.4 Kits
Gel extraction kit for DNA purification and TRI reagent for RNA isolation and the protein estimation kit by BCA method were purchased from Sigma-Aldrich Chemicals Co. (USA). The RevertAid™ Premium First Strand cDNA Synthesis
Material and Methods...

Kit, Maxima® SYBR Green/ROX qPCR Master Mix (2X) and crystal violet staining solution were procured from Fermentas (Thermo Fisher Scientific Inc, USA). Trizol from Gibco-BRL, NY, USA was used for RNA isolation from macrophages.

Table 3.1.1 Enzymes used in the study

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA ligase (10 U/μl), Proteinase K</td>
<td>Bangalore Genei (INDIA)</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5 U/μl)</td>
<td>New England Biolabs (USA)</td>
</tr>
<tr>
<td>MuLV Reverse Transcripase</td>
<td>New England Biolabs (USA)</td>
</tr>
</tbody>
</table>

3.1.5 Strains and plasmid

*E. coli* M15 strain used as host for cloning and expression purpose was procured from Qiagen, Valencia, CA, USA. *M. tuberculosis* H37Rv (ATCC no. 25618) and *M. tuberculosis* H37Ra (ATCC no. 25177) strain, both were a kind gift from Dr. Charu Sharma, Scientist, Drug Target Discovery and Development Division Central Drug Research Institute, Lucknow and Dr. V.M. Katoch, National Institute of leprosy and other mycobacterial diseases, Agra, India respectively. The vector pQE-30 UA (Qiagen, Germany) was used for expression study (Fig. 3.1.1). The characteristics features of the bacterial strains and vectors are given below.

Table 3.1.2 Host strain with their genotype and phenotype

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> M15 strain</td>
<td><em>NalS, StrS, RifS, Thi−, Lac−, Ara+, Gal+</em>, Mtl−, F−, RecA+, Uvr+, Lon+*</td>
</tr>
</tbody>
</table>
Table 3.1.3 Plasmid, pQE-30 UA with their sequence characteristics

<table>
<thead>
<tr>
<th>Sequences Characteristics</th>
<th>Positions of elements in bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector size (bp)</td>
<td>3504</td>
</tr>
<tr>
<td>Start of numbering at Xhol (CTCGAG)</td>
<td>1-6</td>
</tr>
<tr>
<td>T5 promoter/lac operator element</td>
<td>7-87</td>
</tr>
<tr>
<td>T5 transcription start</td>
<td>61</td>
</tr>
<tr>
<td>6xHis tag coding sequence</td>
<td>127-144</td>
</tr>
<tr>
<td>Multiple cloning site I</td>
<td>145-173</td>
</tr>
<tr>
<td>Cloning site for PCR product</td>
<td>174-175</td>
</tr>
<tr>
<td>Multiple cloning site II</td>
<td>176-235</td>
</tr>
<tr>
<td>Lambda 60 transcriptional termination region</td>
<td>251-345</td>
</tr>
<tr>
<td>rrnB T1 transcriptional termination region</td>
<td>1107-1205</td>
</tr>
<tr>
<td>ColE1 origin of replication</td>
<td>1681</td>
</tr>
<tr>
<td>β-lactamase coding sequence</td>
<td>3299-2439</td>
</tr>
</tbody>
</table>

Figure 3.1.1 pQE-30 UA vector map
Material and Methods...

Figure 3.1.2 Multiple cloning site in pQE-30 UA vector

Mice and Cell lines
Inbred strains of pathogen free female BALB/c mice (6-8 weeks old; 20-25 g) were obtained from Animal House Facility, Department of Zoology, University of Delhi, Delhi, India. The animals were reared in uniform hygienic conditions with controlled environment (temp. 20-25°C; and 12h dark/light cycle) following guidelines of Animal Ethics Committee, University of Delhi, Delhi, India. RAW 264.7 cells, an Abelson leukemia virus transformed murine macrophage cell line (ATCC number, TIB-71), were obtained from the American Type Culture Collection (ATCC), USA. These are adherent cells obtained from *Mus musculus* (mouse-Balb/c). RAW 264.7 murine macrophage cell line were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% HEPES, 1% L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin at 37°C with 5% CO₂.

3.1.6 Preparation of growth media
3.1.6.1 Liquid growth media
Middlebrook 7H9 broth base (Hi-Media) was prepared in distilled water and supplemented with 1% glycerol and 0.05% Tween-20. An additional growth supplement OADC (BBL) was added in 2% proportion. Liquid growth media for *E. coli* M15 cells was prepared by dissolving 2.0% Luria-Bertani (LB) (ready-made) in distilled water. The pH of media was adjusted to 7.2 and autoclaved at 15 lbs for 15 min.
3.1.6.2 Solid growth media
Solid growth media was prepared by dissolving 2% Luria-Bertani (LB) medium and 1.5% agar in distilled water. The pH of media was adjusted to 7.2 and autoclaved at 15 lbs (121°C) for 15 min.

3.1.6.3 Preparation of SOB media for ultra-competent cells
This media was prepared by dissolving the following components in 500 ml autoclaved water.
- 5% yeast extract
- 2% tryptone
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl₂
- 10 mM MgSO₄

The pH of the media was adjusted to 7.0 using 5N NaOH and sterilized by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²).

3.1.7 Transformation buffer
Transformation buffer (250 ml) for ultra-competent cells was prepared by adding the following components in autoclaved distilled water.
- 15 mM CaCl₂
- 250 mM KCl
- 10 mM PIPES
- 55 mM MnCl₂

*Note: The pH of the transformation buffer was adjusted to 6.7 using KOH followed by addition of 55mM MnCl₂ and final volume was adjusted.

3.1.8 Preparation of ultra-competent cells
The ultra-competent cells preparation was carried out as follows
- E. coli M15 cells were cultured on LB agar plate at 37°C overnight.
Material and Methods...

- About 5 colonies were picked up and inoculated in 250 ml SOB in 500 ml flask at 19°C with vigorous shaking till OD reached to OD_{600}=0.5.
- The grown culture was kept in ice for 10 min.
- Cultured cells were pelleted at 4000 rpm for 10 min in cold centrifuge.
- The cells were resuspended in 80 ml ice-cold TB and store on ice for 10 min.
- The cells were spun at 4000 rpm for 10 min in cold centrifuge.
- Cells pellet was resuspended again in 20 ml ice-cold TB and 1.4 ml DMSO was added to the cells.
- The cells were aliquot to 100 μl and stored at -80°C.

3.1.9 Preparation of antibiotics/IPTG

The ampicillin was dissolved as 100 mg/ml stock by dissolving 1 g ampicillin in 10 ml of distilled autoclaved water. The kanamycin was dissolved as 30 mg/ml stock in autoclaved water; both were sterilized using micro syringe filters of 0.2 μm size. IPTG was dissolved as 1 M stock in autoclaved water and sterilized using micro syringe filters (0.2 μm size).

3.1.10 BUFFERS (Sambrook et al., 1989)

3.1.10.1 Tris HCl buffer (pH-8.0)

1 M Tris-CI stock was prepared by dissolving 60.5 g Tris base in 450 ml distilled water and pH was adjusted using 6 N HCl. The final volume was adjusted to 500 ml.

3.1.10.2 EDTA (pH 8.0)

EDTA was prepared as 0.5 M stock by dissolving 186.1 g disodium salt of ethylenediamene tetra acetic acid in 450 ml distilled water. The pH of EDTA was adjusted by adding NaOH pellets and was mixed vigorously on magnetic stirrer. The final volume was adjusted to 500 ml.
3.1.10.3 Buffer Used for enzyme assays

3.1.10.3.1 Sodium Acetate Buffer (pH 5.0)
The 1 M stock solution of sodium acetate was prepared and pH was adjusted to 5.0 with acetic acid. This stock solution was used to prepare 0.05 M working buffer of pH 5.0.

3.1.10.3.2 Phosphate Buffer (pH 6.0)
1 M sodium dihydrogen phosphate (NaH$_2$PO$_4$) was prepared and pH was adjusted to 6.0 with 1 M disodium hydrogen phosphate (Na$_2$HPO$_4$). This stock solution was used to prepare 0.05 M of phosphate buffer of pH 6.0.

3.1.10.3.3 Phosphate Buffer (pH 7.0)
1 M Disodium hydrogen phosphate (Na$_2$HPO$_4$) was prepared and pH was adjusted to 7.0 with 1 M sodium dihydrogen phosphate (NaH$_2$PO$_4$). This stock solution was used to prepare 0.05 M of phosphate buffer of pH 7.0.

3.1.10.3.4 Phosphate Buffer (pH 8.0)
1 M Disodium hydrogen phosphate (Na$_2$HPO$_4$) was prepared and pH was adjusted to 8.0 with 1 M sodium dihydrogen phosphate. This stock solution was used to prepare 0.05 M of phosphate buffer of pH 8.0.

3.1.10.3.5 1 M Tris-Cl Buffer (pH 9.0)
The required amount of Tris base was dissolved in distilled water and pH was adjusted to 9.0 using conc. HCl.

3.1.10.3.6 Glycine-NaOH buffer pH (10.0 and 10.5)
Stock solutions of 200 mM glycine and sodium hydroxide were prepared. Glycine stock solution (0.25 ml) was combined with x ml of 0.2 M NaOH to prepare buffer of pH 10.0 and 10.5 and diluted with de-ionized water to make a 100 ml solution. Note: All buffers and solutions were autoclaved at 15 lb for 15 min before use.
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3.1.10.4 TAE buffer (50X)
The TAE buffer used for DNA electrophoresis was prepared as 50X concentrated solution by dissolving 121 g Tris base, 28.8 ml glacial acetic acid and 50 ml of 0.5 M EDTA (pH 8.0). The final volume was adjusted to 500 ml.

3.1.10.5 Buffer used for lysis of cells to release the intracellular protein
The buffer was prepared by using 150 mM NaCl, 0.1% Triton X-100, 1mM EDTA and 50 mM Tris-Cl buffer (pH 8.0).

3.1.10.6 Solubilizing buffer for inclusion bodies
It was prepared by mixing 0.1 M sodium dihydrogen phosphate, 0.01 M Tris-Cl and 6 M guanidine hydrochloride. pH of the buffer was adjusted to 8.0 with 5N NaOH.

3.1.10.7 Buffer used for the purification of intracellular protein under denaturing conditions using Ni-NTA resin

3.1.10.7.1 Buffer A, Equilibration buffer
It was prepared by mixing 0.1 M sodium dihydrogen phosphate, 0.01 M Tris-Cl, 8 M Urea. The pH was adjusted to 8.0 using NaOH.

3.1.10.7.2 Buffer B, Wash buffer
It was prepared by mixing 0.1 M sodium dihydrogen phosphate, 0.01 M Tris-Cl, 8 M Urea. The pH was adjusted to 6.3.

3.1.10.7.3 Buffer C, Elution buffer
It was prepared by mixing 0.1 M sodium dihydrogen phosphate, 0.01 M Tris-Cl, 8 M Urea. The pH was adjusted to 4.5 using HCl.
3.1.11 Preparation of acrylamide solution and SDS-PAGE
The acrylamide solution was prepared as 30% stock by dissolving 29 g acrylamide and 1% bisacrylamide in (29:1) ratio dissolved in 60 ml water. The final volume was adjusted to 100 ml. The prepared solution was filtered through Whatman filter paper No.1. The polyacrylamide gel (12%) was prepared according to Laemmli (1970).

3.1.12 Resolving gel Tris Buffer (pH 8.8)
Resolving gel buffer used in SDS-PAGE was prepared as 1.5 M by dissolving 18.2 g Tris base in 80 ml distilled water, the pH was adjusted using concentrated HCl. The final volume was adjusted with distilled water to 100 ml.

3.1.13 Stacking Gel Tris Buffer (pH 6.8)
The stacking gel buffer was prepared as 1 M stock concentration by dissolving 12.1 g Tris base in 80 ml distilled water. The pH was adjusted with concentrated HCl. The final volume was adjusted to 100 ml using distilled water.

3.1.14 Reservoir Buffer (Tris-glycine buffer 5X)
A 5X stock solution was prepared by dissolving 15.1 g of Tris base and 72 g of glycine in 500 ml distilled, final volume was adjusted to 1000 ml.

3.1.15 Sample Buffer for proteins (5X)
The sample buffer was prepared as 5X concentration by using 250 mM Tris Cl (pH 6.8), 500 mM β-mercaptoethenol, 10% SDS, 0.5% bromophenol blue and 50% glycerol. The sample buffer was finally used depending upon the concentration of proteins.

3.1.16 Loading of proteins samples and SDS PAGE electrophoresis
The gel plates were placed in electrophoresis tank. The upper and lower reservoir of tank was filled with 1X running buffer. The appropriate volume of
sample was mixed with loading buffer (5X) or (1X) depending upon the concentration of protein and was carefully loaded in wells. The protein was separated using SDS polyacrylamide gel electrophoresis according to Laemmli (1970). Electrophoresis was carried out initially at 10 mA and later when protein sample completely entered the stacking gel. The current was increased to 15 mA. The power supply was turned off when tracking dye reached to bottom of gel and gel was taken out of the electrophoresis tank. The gel was carefully removed from the plates and transferred to clean glass tray containing coomassie brilliant blue dye.

3.1.17 DNA Loading dye (6X)
This dye was prepared according to the Sambrook et al., (1989) by dissolving 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water.

3.1.18 Staining solution for PAGE gel
Methanol, acetic acid and distilled water in ratio 5:1:4 with 0.1% Coomassie Brilliant Blue dye were mixed to prepare staining solution.

3.1.19 Destaining solution for PAGE gel
Methanol, acetic acid and distilled water were mixed in 10:7.5:82.5 ratios.

3.1.20 Solution to stop enzyme reaction
1.06 g of sodium carbonate was dissolved in 100 ml of distilled water to make it 0.1 M stop solution.

3.1.21 Agarose Formaldehyde Electrophoresis
RNA electrophoresis under denaturing conditions in 2.2 M formaldehyde was performed according to Maniatis et al. (1982) using the MOPS buffer system. RNA samples were prepared by adding 5 µl of RNA (2.5 µg/µl) to 15 µl RNA
denaturation buffer. 1 μl of 10mg/ml ethidium bromide is added to aid visualization of RNA after electrophoresis.

3.1.21.1 MOPS buffer (10X)
The MOPS buffer used for RNA electrophoresis was prepared as 10X concentrated solution by dissolving 0.2 M MOPS (morpholino-propane sulphonic acid), 50 mM sodium acetate (NaOAC), 5 mM EDTA (pH 8.0) in DEPC H₂O. The final volume was adjusted to 1L with DEPC H₂O. The buffer is adjusted to pH 7.0 with 1M NaOH, sterilized by autoclaving stored at 4°C in dark.

3.1.21.2 RNA Denaturing buffer (1X)
It was prepared by mixing 1.5 ml 10X MOPS buffer (final 1x), 3.5 ml of 40% formaldehyde, 10 ml of 100% deionized formamide. Formamide is deionized by stirring 100ml with approximately 20g of Amberlite MB3 (Merck, Germany) ion exchange resin for 15 minutes.

3.1.21.3 Agarose formaldehyde gel (2.2 M, 1.5%)
1.5 g agarose was dissolved by boiling in 72 ml H₂O in microwave and then cooled to 55°C. 10 ml of 10X MOPS buffer and 18 ml de-ionized formaldehyde were added.

3.1.21.4 RNA Loading dye (10X)
This dye was prepared according to the by dissolving 0.25% bromophenol blue, 0.25% xylene cyanol FF, 50% glycerol and 10 mM EDTA (pH 8.0) in water.

3.1.22 Ethidium Bromide Solution
10 mg/ml stock solution was prepared in sterilized distilled water and used at a final concentration of 0.5 μg/ml.
Table 3.1.4 Oligonucleotide sequences used in cloning of Rv1923 and Rv2485c genes and for amplification of Rv1922, 16S RNA (Endogeneous Control), iNOS and GAPDH (Endogeneous Control)

<table>
<thead>
<tr>
<th>OligoName</th>
<th>Oligo Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rv1923- Forward primer</td>
<td>5'-TTG GAT GTA GCG GGG CTA CC-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-GAG CAG TTA TGC ACC CGA GG-3'</td>
</tr>
<tr>
<td>2. Rv2485c-Forward primer</td>
<td>5'-TGC ACA TCG CCA GCG TGA CTT-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-GAA CGG CCA AGC TCA GCT-3'</td>
</tr>
<tr>
<td>3. Rv1922- Forward Primer</td>
<td>5'-GCGGATCTGGCATCCGGC-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-ATGCGCGGTGGACATCT-3'</td>
</tr>
<tr>
<td>4. 16S- Forward Primer</td>
<td>5'-AGA GTT TGA TCC TGG CTC AG -3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-TAC GGY TAC CTT GTT ACG ACT T-3'</td>
</tr>
<tr>
<td>5. iNOS- Forward Primer</td>
<td>5'-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3'</td>
</tr>
<tr>
<td>6. GAPDH- Forward Primer</td>
<td>5'-CTC GTG GAG TCT ACT GTT GT-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-GTC ATC ATA CTT GGC AGG TT-3'</td>
</tr>
</tbody>
</table>

Note: All primers diluted as 10 μmolar concentration and finally used as 1 μmolar/25 μl of PCR reaction.

3.2 METHODS

3.2.1 Mycobacterium tuberculosis H37Rv DNA isolation
All manipulations of live M. tuberculosis were carried out under Biosafety Level 3 conditions. M. tuberculosis H37Rv strains were grown in Middlebrook 7H9 liquid medium (Hi-Media) supplemented with 1% glycerol, 0.05% Tween 20 and 2% OADC. Bacteria were grown at 37 °C. Genomic DNA of M. tuberculosis H37Rv was extracted at JALMA, Agra, India. It was extracted from harvested log phase culture by the method of Van Soolingen and Hermans (1995).

3.2.2 Quantification of the DNA
Mycobacterium tuberculosis H37Rv DNA was quantified spectrophotometrically at 260 nm and 280 nm. A260/280 was evaluated to know purity of DNA using Sambrook et al., 1989.
3.2.3 Designing of primer sets

Primers were designed using Rv1923, Rv2485c and Rv1922 gene sequences reported at NCBI gene bank (Benson et al., 2009; Sayers et al., 2009) site (http://www.ncbi.nlm.nih.gov/nuccore) with accession number NC_000962; Region: 2175173..2176513 for Rv1923, Region: complement 2792723..2793988 for Rv2485c and Region: 2174067..2175182 for Rv1922. The Primers were checked for their hairpin loop formation, GC contents and for primer dimer formation using IDT (Integrated DNA Technologies, Inc., U.S.A.) tools for oligo analysis available online.

3.2.4 Polymerase chain reaction

Cloning of Rv1923 and Rv2485c was carried out following PCR based approach.

3.2.5 Standardization of PCR Reaction

The PCR reaction was standardized using gradient temperature for annealing i.e. 56/62°C.

Table 3.1.5  Standard PCR reaction mix composition for cloning of Rv1923, Rv2485c and Rv1922 genes

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Final concentration</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer</td>
<td>1X</td>
<td>10 μL</td>
</tr>
<tr>
<td>MgCl2 (25 mM)</td>
<td>1.5 M/2.0 mM</td>
<td>3.1μL</td>
</tr>
<tr>
<td>dNTPs Mix (25 mM)</td>
<td>1 mM</td>
<td>4.0μL</td>
</tr>
<tr>
<td>Forward primer (10μm)</td>
<td>1μM</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>Reverse primer (10μm)</td>
<td>1μM</td>
<td>4.0μL</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5 U/μl)</td>
<td>1 unit</td>
<td>1.0μL</td>
</tr>
<tr>
<td>Template DNA</td>
<td>0.1 μg</td>
<td>2.0 μL</td>
</tr>
<tr>
<td>Triple autoclaved distilled water</td>
<td>72 μl</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>100 μL</strong></td>
<td></td>
</tr>
</tbody>
</table>
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Table 3.1.6 Standard PCR reaction conditions for amplification of Rv1923

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>4 min.</td>
<td>1X</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62°C</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>2 min.</td>
<td>30X</td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>7 min.</td>
<td>1X</td>
</tr>
<tr>
<td>4</td>
<td>4.0°C</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1.6 Standard PCR reaction conditions for amplification of Rv2485c gene

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>4 min.</td>
<td>1X</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>2 min.</td>
<td>30X</td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>7 min.</td>
<td>1X</td>
</tr>
<tr>
<td>4</td>
<td>4.0°C</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.1.6 Standard PCR reaction conditions for amplification of Rv1922

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>4 min.</td>
<td>1X</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57°C</td>
<td>40 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>2 min.</td>
<td>30X</td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>7 min.</td>
<td>1X</td>
</tr>
<tr>
<td>4</td>
<td>4.0°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1.7 Ligation of the PCR amplified product

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA ligase buffer (2X)</td>
<td>5.0</td>
</tr>
<tr>
<td>PCR product (0.1 µg/µl)</td>
<td>2.5</td>
</tr>
<tr>
<td>pQE-30 UA vector (50 ng/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>T4 DNA ligase (10 U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

**Note:** Ligation was carried out at 16°C for 16h.

3.2.6 Amplification of Rv1923 and Rv2485c genes

Amplification of lipase gene was carried out using designed set of specific primers according to above reaction conditions. Amplified samples were separated on a 1.2% agarose gel containing ethidium bromide in 1X TAE buffer. After electrophoresis, the gel was viewed by the Biorad electrophoresis documentation and analysis system (USA).
3.2.7 Cloning of the Rv1923 and Rv2485c genes into pQE-30 UA vector
The PCR amplified products were gel eluted using gel extraction kit (Sigma Chemicals Co. USA) and ligated to pQE-30 UA vector as given in Table 3.1.7

3.2.8 Transformation
The transformation of the ligated mix was carried out in ultra-competent E. coli M15 cells as follows.
- The cells stored in -80°C were taken out and kept on ice.
- The 5 µl ligated mix was added in 100 µl ultra competent cells.
- The cells containing ligated mix were kept in ice for 45 min.
- Heat shock was given at 42 °C for 90 s and again kept on ice for 2 min.
- 895 µl LB media was added in the reaction tube and grown at 37 °C for 2 h.

3.2.9 Confirmation of recombinant clones
Recombinant clones were confirmed by gel electrophoresis of recombinant and non-recombinant plasmids and by colony PCR.

3.2.9.1 Plasmid DNA isolation
Plasmid DNA was isolated according to the method by Sambrook et al. (1989).

3.2.9.2 Colony PCR
The recombinant pin point colonies (E. coli M15 cells) were taken out aseptically using sterile toothpick and added in autoclaved microcentrifuge tubes containing 50 µl colony lysis buffer (0.1% Tween 20 in TE buffer, pH 8.0) and vortexed. The microcentrifuge tube containing cells were then kept on boiling water bath for 10 min followed by cooling on ice for 2 min. The centrifugation was carried out at 10,000 rpm for 2 minutes. 1 µl lysate was used as template for PCR amplification of the lipase genes. The reaction mix preparation (except primers) and conditions for amplification were similar as used in primary PCR.
Table 3.1.10 Oligo nucleotide sequence used to check orientation of the gene in pQE-30 UA cloning

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward sequencing primer</td>
<td>5'- CCCGAAAAGTGCCACCTG -3'</td>
</tr>
<tr>
<td>Reverse sequencing primer</td>
<td>5'- GTTCTGAGGTCATTACTGG-3'</td>
</tr>
<tr>
<td>Forward primer (For Rv1923)</td>
<td>5'- TTG GAT GTA GCG GGG CTA CC -3'</td>
</tr>
<tr>
<td>Forward Primer (For Rv2485c)</td>
<td>5'- TGC ACA TCG CCA GCG TGA CTT 3'</td>
</tr>
</tbody>
</table>

3.2.10 Sequencing and analysis of the Rv1923 and Rv2485c genes

Plasmid DNA was extracted from both the clones were sequenced using sequencing primers for pQE-vectors. All nucleotide sequencing reactions were carried out by using an automated AB1 3100 genetic analyzer (Bangalore Genei) that uses fluorescent label dye terminator based on Sangers sequencing method. The full length gene sequence alignment was carried out using different tools available at European Bioinformatics Institute (EBI) and National Center for Biotechnology Information (NCBI) for protein and nucleotide analysis (Altschul et al., 1990). BlastN and BlastX were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to search homology of the lipase gene against the gene database. Multiple sequence alignment was carried out using Clustal W (Thompson et al., 1994). The signal peptide present in amino acids sequence of the protein was determined using SignalP4.0 server, Petersen et al., 2011 (http://www.cbs.dtu.dk/services/ SignalP/). Secondary structure for the mature polypeptide was carried out with SOPMA (Geourjon and Deleage, 1995) and the pi of the mature polypeptide was calculated using ScanSite pi/Mw (http://scansite.mit.edu/calc_mw__pi.html). The protein structures of Rv1923 and Rv2485c were predicted by homology modeling using online software, Phyre (Kelley and Sternberg, 2009) and image was produced and labeled by PyMol (DeLano, 2002).
3.2.12 Expression of recombinant protein
The E. coli M15 cells carrying recombinant plasmid pQE-30 UA with lipase insert were initially cultured overnight at 37°C in 5 ml liquid LB media containing ampicillin (100μg/ml) and kanamycin (30 μg/ml) in orbital shaker. Next day 1% of overnight grown culture was inoculated in a flask containing 1L LB with ampicillin (100 μg/ml) and kanamycin (30 μg/ml). The culture was grown again at 37°C in orbital shaker till OD was reached to log phase i.e. A600=0.5 and finally induced with 0.5 mM IPTG for four hours at 37°C.

3.2.13 Purification of recombinant protein
3.2.13.1 Purification of inclusion bodies (Rv1923 & Rv2485c)
Cells were harvested at 4°C by centrifugation at 8000 g for 15 min and resuspended in cold lysis buffer (50 mM TrisCl, pH 8.0, 0.1% (v/v) triton X-100, 150 mM NaCl and 1 mM EDTA) at 5ml/g wet weight and stored overnight at -80 °C. The cell suspension was thawed on ice for 1 h and 20 mM MgSO4 (final concentration) was added. Cells were disrupted by ultrasonication (10× with a 15 s cycle) using a Pharmacia Biotech Sonifier. Inclusion bodies were separated from the cell extract by centrifugation at 18,000 g for 20 min. The pellet was solubilized by stirring at 4°C overnight in a 40 mL solution containing 10 mM Tris/HCl, pH 8.0, 100 mM sodium dihydrogen phosphate and 6 M guanidine hydrochloride. The insoluble debris were separated from solubilized protein by centrifugation at 14,000 g for 20 min at 4 °C. The supernatant was loaded (3 mL min⁻¹) onto a Ni²⁺-agarose column (1 mL resin for 5 mg of recombinant protein) previously equilibrated with buffer A (10 mM Tris/HCl, pH 8.0, 100 mM sodium dihydrogen phosphate and 8 M urea). The column was then washed using five column volumes of buffer B (10 mM Tris/HCl, pH 6.3, 100 mM sodium dihydrogen phosphate and 8 M urea). Enzyme was eluted with buffer C (10 mM Tris/HCl, pH 4.5, 100 mM sodium dihydrogen phosphate and 8 M urea); fractions of eluted peaks containing purified Rv1923 and Rv2485c were analyzed by SDS/PAGE (Laemmli et al., 1970) and pooled.
3.2.13.2 Refolding of inclusion bodies by dialysis
Refolding was done with chaotropic agents' concentration gradient dialysis. The solution of denatured protein (1.5 mg/ml) was dialyzed against 2 L of freshly made 6, 4, 2, 1, 0.5, and 0 M urea, respectively, with 10 mM sodium phosphate buffer (pH 8.0). With each concentration, the protein was dialyzed 6 h at 4 °C.

3.2.14 Standard curve for protein estimation
The BCA working reagent was prepared by mixing 50 parts of Reagent A (Bicinchoninic acid solution) with 1 part of Reagent B (Copper (II) Sulfate Pentahydrate 4% Solution, provided in BCA assay protein kit, Sigma). A stock solution of BSA, 1.0 mg/ml was diluted to get solution of different concentration of 50, 100, 150, 200, 250, 500, 750 μg/ml respectively. 20 parts of the BCA (Bicinchoninic Acid Solution) working reagent were then mixed with 1 part of a BSA of different concentrations. The absorbance $A_{562}$ was recorded and standard curve was drawn by plotting a graph between protein concentration vs absorbance (562 nm).

3.2.15 Standard curve for p-Nitrophenol
A stock solution of p-Nitrophenol (10 mM) was prepared in ethanol and further dilution of different concentration was prepared ranging from 0.02 mM to 1.0 mM. The absorbance $A_{420}$ was recorded and standard curve was drawn, by plotting the optical density values against p-Nitrophenol concentration. The concentration constant for the p-nitrophenol was calculated to 0.067 μmoles/ml.

3.2.16 Standard curve for Nitric oxide
The dilution of different concentration was prepared ranging from 3.125 μM/ml to 100 μM/ml. The absorbance $A_{540}$ was recorded and standard curve was drawn, by plotting the optical density values against NO concentration.
Material and Methods...

3.2.17 Enzyme assay
The enzyme activity was determined according to the modified method of Sigurgisladottir et al., (1993). To 0.8 ml of 0.05 M phosphate buffer (pH 8.0), 0.1 ml enzyme (appropriately diluted) and 0.1 ml of 0.002 M p-nitrophenyl palmitate was added. The reaction was carried out at 40°C for 15 min, after which 0.25 ml of 0.1 M Na₂CO₃ was added. The mixture was centrifuged and the activity was determined by measuring absorbance at 420 nm in UV/Vis spectrophotometer (JENWAY 6505 UK). One unit of enzyme activity is defined as the amount of enzyme, which liberates 1 μmole of p-nitrophenol from pNP-palmitate as substrate per min under standard assay conditions. The total enzyme activity was expressed in Units and specific activity was expressed as U/mg of protein.

3.2.18 Biochemical characterization of Rv1923 and Rv2485c enzymes
3.2.18.1 Substrate Specificity
The substrate specificity of Rv1923 and Rv2485c enzymes was carried out using pNP ester of following chain length, pNP acetate (C₃), pNP butyrate (C₄), pNP deconate (C₁₀), pNP-palmitate (C₁₂), pNP myristate (C₁₄), pNP palmitate (C₁₆), pNP stearate (C₁₈) (Sigma Chemical Co. USA), dissolved as 10 mM stock in ethanol and used as 2.0 mM concentration. The assay was performed (for both Rv1923 and Rv2485c enzymes) according to our standard assay method at 40°C and pH 8.0.

3.2.18.2 Effect of temperature on enzyme activity and stability
The enzymatic activity of lipase was studied at different temperatures (20-90°C). The assay was carried out at pH 8.0. Thermostability of the recombinant enzyme was carried out by pre-incubating the enzyme for 30 min (20-90 °C) followed by cooling on ice for 15 min, before the enzyme assay. The enzyme without incubation was taken as control (100%) and reaction mix without enzyme served as blank.
To further probe the time dependent thermostability of the lipase at 50 °C and 55 °C, the lipase was pre-incubated for 15, 30, 45, 60 and 75 min at above said temperatures. The heat treated enzyme was cooled on ice for 15 min before enzyme assay. The reaction mix without enzyme served as blank. The reaction mix containing enzyme without incubation was taken as control (100%).

3.2.18.3 Effect of pH on enzyme Activity and stability
The lipase activity was assayed at different pH (4.0-10.5). The enzyme stability at different pH was studied by pre-incubating 0.1 ml of enzyme with 0.1 ml of 50 mM buffer for one hour at room temperature. The assay was carried out with appropriate diluted enzyme according to the standard assay conditions. The enzyme without incubation was taken as control (100%).

3.2.18.4 Kinetic study of Rv1923 & Rv2485c
The effect of substrate pNP-palmitate (0.1 mM-5 mM) on the rate of enzyme reaction was carried out using standard assay method. The Michaelis–Menten constant ($K_m$) and maximum velocity for the reaction ($V_{max}$) with pNP-palmitate as substrate were calculated by Lineweaver-Burk plot, $k_{cat}$ and $k_{cat}/K_m$ were calculated for Rv1923 and Rv2485c enzymes.

3.2.18.5 Effect of Different additives and chemical modifiers on Rv1923 and Rv2485c activity
The effect of various additives PMSF, serine, DEPC, β-ME and SDS (0.1 and 1.0 mM) were studied by addition of these additives separately into the reaction mix. The activity was measured according to standard assay conditions. The sample without any additive was taken as control (100%). The reaction mix with respective additives but without enzyme served as blank.
Material and Methods...

3.2.19 Biophysical characterization of Rv1923

3.2.19.1 Circular dichroism spectroscopy of native protein
Far UV-CD spectra were collected at 25°C on a Chirascan (Applied Photophysics) spectrometer calibrated using 10-camphorsulfonic acid. The protein solutions were dialyzed extensively against 10 mM potassium phosphate (pH 8.0) prior to measurements. Spectrum of purified, refolded Rv1923 in 10 mM phosphate buffer, pH 8.0, was recorded as an average of 6 spectral scan in the far-UV region from 195 to 260 nm using a cuvette with a path length of 0.2 cm. Following were the instrument parameters: instrument sensitivity, 1 millidegrees; response time, 2 s; scan speed, 30 nm/min. The mean residue ellipticity (MRE, deg cm²dmol⁻¹ residue⁻¹) was calculated from the baseline-corrected spectrum.

3.2.19.2 Thermal and pH Stability by Circular Dichroism
The effect of temperature and pH on enzyme conformation (secondary and tertiary) was monitored by CD spectroscopy. Thermal and pH stability was assessed in the Far UV range from wavelength 195 to 260 nm with increasing temperature from 20-90°C and at pH 2-9 respectively. Thermal denaturation was also monitored by circular dichroism (CD) at 222 nm at different temperatures. Also, data obtained from the CD spectra recorded in the 195–260 nm range were used to calculate the secondary structure composition of the protein using the web-based program K2D (Andrade et al., 1993 and Merelo et al., 1994).

3.2.19.3 Urea denaturation
Rv1923 was incubated O/N with 1M to 8M urea concentration. Circular dichroism (CD) was monitored at 222 nm at different urea concentration 1M to 8M.
3.2.20 Demonstration of the probable role of Rv1923 and Rv2485c in the modulation of immune response

3.2.20.1 Immunization, Isolation of Immunoglobulins and Antibody titre determination

3.2.20.1.1 Immunization

BALB/c mice each were immunized three times (one primary and two boosters) with the 50 μg protein (Rv1923 or Rv2485c) mixed with FCA (primary) or FIA (boosters) at weeks 0, 2 and 4, respectively. Immunizations were carried out by intraperitoneal injection of the prepared emulsion. A total of 3 animals were immunized for each protein. And the immunization was repeated twice with different batch of purified proteins.

3.2.20.1.2 Sera collection

Mice were bled through the retro-orbital plexus and the sera were separated by centrifugation for the immunoglobulin assay.

3.2.20.1.3 Antibody assay

Serum anti-Rv1923 or anti-Rv2485c antibody (IgG) titers were measured by ELISA using purified Rv1923 or Rv2485c protein as the solid phase antigen. Briefly, ELISA plates (96-well, U bottom by Tarson, India) were coated overnight with 200μl (5 μg/ml) of purified proteins (Rv1923 or Rv2485c) in phosphate buffer saline (PBS), and non-specific sites were then blocked with 1% bovine serum albumin in PBS. After washing twice with PBS having 0.5% Tween-20, serum samples diluted in PBS (pH 7.4) were added to the wells. After overnight incubation at 4°C in the cold-room, the plates were successively washed with PBS and Tween-20 and incubated with 1: 2000 dilution of horseradish peroxidase, HRP-conjugated goat antimouse IgG antibodies (Bangalore Genei) for 2h at room temperature. Plates were washed again, and 100 μl of orthophenylenediamine dihydrochloride solution (OPD) (1 mg/ml) in 0.05 M
citrate buffer (pH 5.0) and 2 μl 30% H₂O₂ were added. Absorbance at 490nm was recorded after the addition of 10 μl of oxalic acid as stop solution.

3.2.20.2 Western Blotting to detect localization of Rv1923 and Rv2485c

3.2.20.2.1 Preparation of mycobacteria cellular fraction
*M. tuberculosis* H37Ra allowed to reach mid-exponential phase in 200 ml Middlebrook 7H9 liquid medium (Hi-Media) supplemented with 1% glycerol, 0.05% Tween 20 and 2% OADC. Cells were collected by centrifugation at 10,000 × g for 20 min and the culture supernatant was saved for concentration following a procedure modified from Dubos et al. (1995). The culture supernatant was passed through a 0.22-μm (pore-size) bottle filter (Sigma-Aldrich, USA) and the filtrate was concentrated 100-fold in a centrifugal filter unit with a 10,000-MW cutoff membrane (Amicon, Inc.). Pelleted cells were washed, resuspended in PBS and mixed with equal volume of 2x SDS loading dye.

3.2.20.2.2 SDS-PAGE and Western blotting
Whole cell pellet and culture filterate were separated by SDS-PAGE (12% gel) along with purified Rv1923 or Rv2485c protein. Proteins from gels were transblotted overnight onto nitrocellulose membranes (Protran, Sigma, USA), which were then blocked for 1 h with PBS containing 5% powdered skim milk and 0.02% sodium azide. Anti-Rv1923 or anti-Rv2495c antibodies used were diluted into PBS containing 0.25% powdered skim milk with 0.02% sodium azide. Membranes were rocked at 4°C for 8 h with primary antibodies, washed and then incubated with secondary antibodies, HRP-conjugated goat anti-mouse IgG antibodies (Bangalore Genei) at 1:2,000 dilutions overnight at 4°C. Membranes were washed with PBS and developed with chromogenic substrate, 100 μl of orthophenylenediamine dihydrochloride solution (OPD) (1 mg/ml) in 0.05 M citrate buffer (pH 5.0) and 2 μl 30% H₂O₂ were added.
3.2.20.3 Expression of Rv1922-LipD operon genes (Rv1923 and Rv1922) and Rv2485c in *M. tuberculosis* H37Ra in response to stress stimuli

3.2.20.3.1 Stress stimulation to *M. tuberculosis* H37Ra

*M. tuberculosis* H37Ra allowed to reach mid-exponential phase in 200 ml Middlebrook 7H9 liquid medium (Hi-Media) supplemented with 1% glycerol, 0.05% Tween 20 and 2% OADC. The cells were grown for 7 days (*A*<sub>600</sub> 1.5-1.6), were divided into four 50 ml aliquots and harvested by centrifugation at 6000xg for 10 min at room temperature. The bacterial pellet was resuspended in different media for exposure to stress conditions as follows, including oxidative stress (5 mM H<sub>2</sub>O<sub>2</sub>), acidic stress (pH 4.5) and nutrient stress (1X phosphate buffer saline).

3.2.20.3.1.1 Acidic stress

The bacterial pellet was resuspended in Middlebrook 7H9 liquid medium (pH 4.5) supplemented with 1% glycerol, 0.05% Tween 20, 2% OADC and 5 mM H<sub>2</sub>O<sub>2</sub> and kept at 37 °C without shaking. After 6 h of acid shock, the culture from each flask was pelleted. RNA isolated from the culture grown in Middlebrook 7H9 liquid medium at pH 7.2 was used as a control or positive calibrator for relative expression analysis.

3.2.20.3.1.2 Nutrient starvation

The Cells were washed twice with PBS with Tween 80 (0.05%, v/v), pH 7.2 and resuspended in 100 ml PBS-Tween (pH 7.2). The culture was incubated at 37°C without shaking. Cells were harvested at 0 and 9 h. Cells harvested at 0 h served as a control (Betts *et al.*, 2002).

3.2.20.1.3 Hypoxia conditions.

The bacterial pellet was resuspended in Middlebrook 7H9 liquid medium supplemented with 1% glycerol, 0.05% Tween 20, 2% OADC and 5 mM H<sub>2</sub>O<sub>2</sub>.
Material and Methods...

The culture was transferred to sealed rubber-capped tube and kept standing at 37°C. For control samples, the pellet was resuspended Middlebrook 7H9 liquid medium without H2O2 and the tube was not sealed and were kept at 37 °C with periodic exposure to air by shaking.

3.2.20.3.2 RNA isolation
Total RNA was isolated from all the stress induced and controls *M. tuberculosis* H37Ra strains using TRI-Reagent as per Sigma-Aldrich manual. Briefly, bacterial cells were pelleted at 10000 rpm for 5 min. The pellets were resuspended in 1 ml TRI-reagent (each) and were made homogenous by pipeting and kept at RT for 30 min. Then 200μl ice cold chloroform was added and mixed properly followed by centrifugation at 13000 rpm for 20 min at 4°C. The above aqueous clear layer containing RNA were transferred into fresh eppendorfs and 600 μl of ice cold isopropanol was added and kept in -20°C overnight. Next day, the samples were centrifuged at 13000 rpm for 20 min .The supernatants were discarded and the pellet obtained was washed with 70% ethanol (with DEPC water). The pellets were dried and redissolved in DEPC water. Each RNA sample was treated with RNase-free DNase I (Fermentas). Isolated RNA was analyzed on agarose gel as well as spectrophotometrically (A260/A280) for checking the quality and quantity, respectively. One volume of the RNA loading dye was mixed with one volume of RNA sample from each set. These were heated at 70°C for 10 min. Chilled on ice for 3 min and then loaded onto the agarose gel.

3.2.20.4 Expression of lipase genes
First strand cDNA was synthesized from 1.5 μg of total cellular RNA from stress induced *M. tuberculosis* H37Ra in a 20 μl reaction volume using RevertAid first strand cDNA synthesis kit (Fermentas). The protocol provided by manufacturer was followed. The synthesized cDNA was stored at -20°C.
Material and Methods...

3.2.20.4.1 Relative Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

One µl of this cDNA was amplified in a total volume of 25 µl PCR reaction mixture containing 5 µl 10X reaction buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% Nonidet P40], 0.2 mM of each dNTP, 2.5 mM MgCl₂, 1 unit Taq DNA polymerase and 10 pmol of adequate primers (for Rv1922, Rv1923 and Rv2485c genes). 16S RNA was used as house-keeping gene (endogenous control). The amplified gene product was checked on agarose gel (1%). The relative levels of mRNA expression of test genes were obtained by calculating the ratio of band intensity of respective gene for each sample separately to intensity of control band intensity.

\[
\text{mRNA level of test gene} = \frac{\text{Test gene band intensity of a sample}}{\text{Control band intensity of the same sample}}
\]

3.2.20.4.2 Quantitative Real-time RT-PCR (qRT-PCR) analysis

Transcription of Rv1923 and Rv2485c genes were quantified using qRT PCR. qRT-PCR was performed with cDNA corresponding to 50 ng RNA using Fermentas Maxima SYBR Green/ROX qPCR Master Mix (2X). Reaction master mix was prepared by adding the following components for each 25 µl reaction at room temperature. The experiment was set up following PCR program on The LightCycler® 480 Real-Time PCR System (Roche Applied Science). Amplification was performed in triplicate wells for each sample analysed; a control reaction with no template (water) was run with all reactions. In each set of reactions, 16S rRNA was used as a reference gene for normalization of cDNA amount. The specificity of the reactions was verified by melting curve analysis. Relative quantification analysis was done using an efficiency-calibrated model (Pfaffl, 2001), and the normalized ratio was calculated as:
Material and Methods...

\[ \text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}}}{(E_{\text{reference}})^{\Delta \text{CP}_{\text{reference}}}} \]

Where \( \text{CP} \) = crossing point, \( \Delta \text{CP}_{\text{target}} = \text{CP}_{\text{control}} - \text{CP}_{\text{sample}} \), \( \Delta \text{CP}_{\text{reference}} = \text{CP}_{\text{control}} - \text{CP}_{\text{sample}} \), \( E_{\text{target}} \) = amplification efficiency of target gene, and \( E_{\text{reference}} \) = amplification efficiency of reference gene.

Table 3.1.5 Real-time RT-PCR reaction mix composition for Rv1923 and Rv2485c

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Final concentration</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SYBR Green Master Mix</td>
<td>1X</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Forward primer (10 µm)</td>
<td>0.4 µM</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Reverse primer (10 µm)</td>
<td>0.4 µM</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>0.5 µg</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td></td>
<td>10.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td><strong>25 µl</strong></td>
</tr>
</tbody>
</table>

Table 3.1.6 A three step cycling protocol for Real-time RT-PCR

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50°C</td>
<td>2 min.</td>
<td>1X</td>
</tr>
<tr>
<td>2</td>
<td>95°C</td>
<td>10 min.</td>
<td>1X</td>
</tr>
<tr>
<td>3</td>
<td>95°C</td>
<td>15 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>30 min.</td>
<td>40X</td>
</tr>
</tbody>
</table>

3.2.20.4.3 Statistical analysis

CP values for each run were used to calculate mean CP with SD (Standard Deviation) and SE (Standard Error). The normalized ratio was calculated using
the efficiency-calibrated model. The expression ratio was calculated from triplicate normalized ratios for each gene with SD.

3.2.21 Cytotoxicity assay for Rv1923 and Rv2485c protein on murine macrophages

The *in vitro* effect of Rv1923 or Rv2485c on Raw 264.7 cells was determined by measuring tetrazolium salt, 3-(4-5-dimethylthiozol-2-yl) 2-5-diphenyl-tetrazolium bromide (MTT) dye absorbance of living cells (Mosmann, 1983). RAW 264.7 cells (1 X 10⁴ per well) were incubated in triplicate in a flat-bottomed 96 microtitre plate. Following an adherence period, the medium was changed and cells were treated with Rv1923 or Rv2485c proteins (with doses-12.25 µg/ml, 25 µg/ml or 50 µg/ml) in the presence or absence of LPS for 24h at 37°C. Raw 264.7 cells cultured without Rv1923 and Rv2485c were used as control. Two hours prior to termination, 20 µl MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37°C, the culture supernatants were removed and 0.1 ml of extraction buffer (20% SDS, 50% dimethylformamide) was added; incubation was continued overnight at 37°C; and then the optical density (OD) at 570 nm was measured by means of a 96-well multiscanner auto reader (Dynatech MR5000, Chantilly, VA).

Alternatively, instead of adding MTT for viability assay, we fixed the adherent macrophages in methanol and then stained in crystal violet for 5 minutes. Washed 3X with tap water. Flicked the plate and completely air dried it before adding 50% Glycerol. After adding glycerol, we observed the plate under inverted microscope (Olympus, Germany) to see the morphological and numerical change in macrophages upon various treatments.
3.2.22 Effect of Rv1923 and Rv2485c on NO production and iNOS expression in murine macrophages

3.2.22.1 Cell culture and activation of macrophages

The murine macrophage cell line, Raw 264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum, 1% HEPES, 1% L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin. The cells were maintained at 37°C humidified atmosphere of 5% CO₂ and were fed every 3-4 days. Cells were grown to approximately 60% confluency in culture flasks.

Raw 264.7 murine macrophages were allowed to adhere for 60 min at 5×10⁵ cells per well in 96-well tissue culture plate. Adhered monolayer of cells were incubated with medium alone or containing 2 μg/ml lipopolysaccharide, LPS (Sigma, USA) together with or without different doses of Rv1923 or Rv2485c (12.50 μg/ ml, 25 μg/ml or 50 μg/ml) for 24 h. Adherence as well as incubation steps were preceded at 37°C with 5% CO₂. The supernatants (conditioned medium) of the 96-well plate were collected and centrifuged at 250g for 10 min, followed by passage through a 0.22 μm membrane filter. These cell-free supernatants were used for the determination of nitrates by Griess reaction as described below.

3.2.22.2 Nitrite assay for estimation of nitric oxide production

The concentration of stable nitrite, an end product from nitric oxide generation by effector macrophages was determined by the method of Ding et al. (1998), based on Griess reaction. Briefly, 100 μl of culture supernatants were incubated with an equal volume of Griess reagent (one part of 1% sulphanilamide in 2.5% phosphoric acid (H₃PO₄) plus one part of 0.1% naphthyl-ethylene-diamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo) in distilled water; two parts being mixed together within 12 h of use and kept chilled) at room temperature for 10 min in a 96 well plate. The absorbance at 540 nm was then determined on Emax microplate reader (Molecular Devices, USA). Nitrite content (μmol/10⁵
cells) was quantified by extrapolation from a sodium nitrite standard curve. Experiments were done in triplicate for at least three times.

3.2.22.3 Expression analysis of iNOS in macrophages by Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from macrophage cells incubated with purified Rv1923 or Rv2485c was isolated using cold Trizol (Gibco-BRL, NY, USA) according to the manufacturer’s instructions. From each sample, 1 µg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1mM dNTP, and oligo (dT12-18) 0.5 µg/ml. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, GAPDH (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 µl containing (final concentration) 1 unit of Taq DNA polymerase, 0.2mM dNTP, 10x reaction buffer, and 100 pmol of forward and reverse primers. After initial denaturation for 2 min at 95°C, thirty amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60 °C annealing, and 1.5 min 72°C extension). After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation. The band intensity was quantified by densitometric analysis.

3.2.22.4 Phagocytosis assay

Raw 264.7 cells (5×10^5 cells per well) were plated in 24-well tissue culture plate and pre-incubated with Rv1923 or Rv2485c protein (5 µg/ml, 10 µg/ml, 20 µg/ml) for 2 h at 37°C in the presence of 5% CO2. Phagocyte culture in medium alone (without lipase) was considered as control and cells treated with LPS (2 µg/ml) alone was considered as positive control. After incubation of cells with Rv1923 or Rv2485c for 2 h, cells were treated with LPS for next 90 min or untreated (used for phagocytosis without LPS treatment).
Material and Methods...

Heat-killed yeast cells (*Saccharomyces cerevisiae*) were used for phagocytosis. Cells on treated plates were washed with PBS and 200 µl of heat-killed yeast cell suspension (3 mg/ml in PBS) was loaded on to the respective well. The phagocytosis was carried out for 2 h at 37°C in the presence of 5% CO₂. After incubation for 10 min cells were washed three times with 200 µl PBS to remove the non-phagocytosed yeasts. After phagocytosis, the medium was removed and 100 µl of ice-cold methanol was added to each well. After 30 min of incubation at room temperature, methanol was removed and the wells were washed three times with 200 µl of PBS. A 1:20 dilution of crystal violet (Fischer scientific, USA) was added to each well for 30 min. Then, crystal violet staining solution was removed and 100 µl of PBS was added to each well. Using inverted microscope (Olympus, Germany), the total number of macrophages and the number of macrophages with intracellular yeast were counted. The phagocytosis percentage was calculated as (the number of infected macrophages/the number of total macrophages) ×100.