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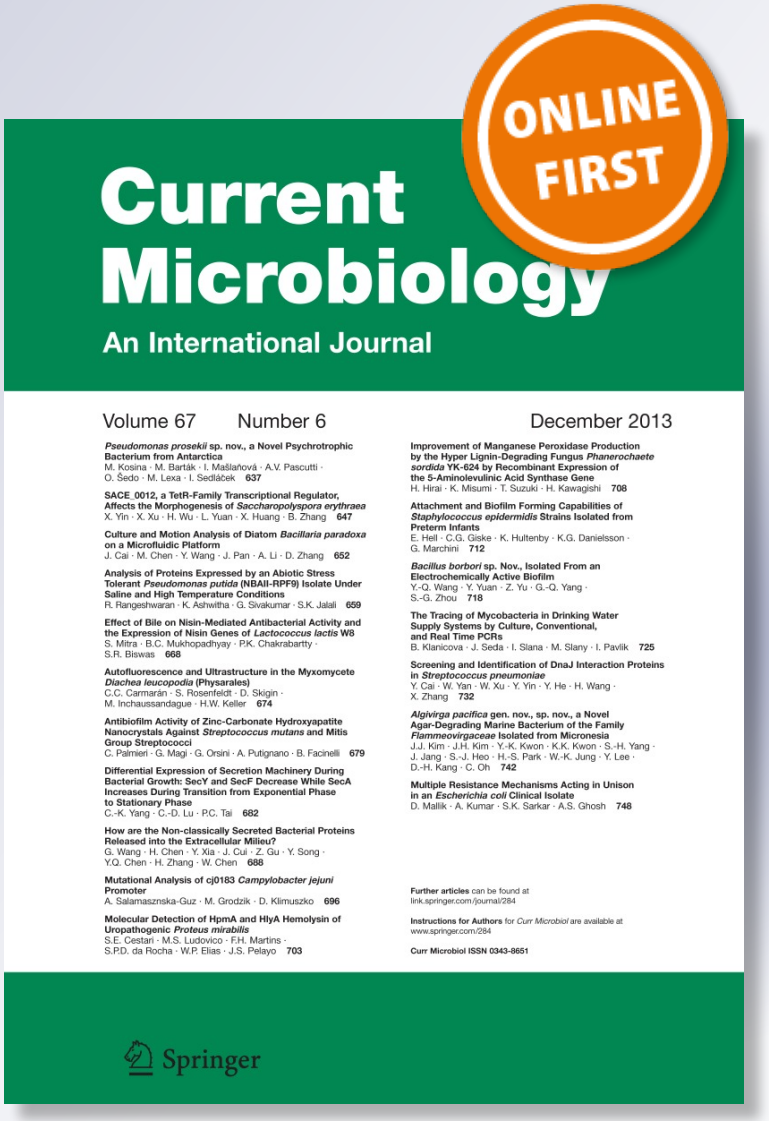

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Molecular Characterization of Oxidative Stress-Inducible LipD of *Mycobacterium tuberculosis* H37Rv

Gurdyal Singh · Stuti Arya · Arbind Kumar · Dominic Narang · Jagdeep Kaur

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Abstract The *Mycobacterium tuberculosis* has developed intricate strategies to evade the killing of microorganism and support its survival in phagocytes. The genome sequence of bacterium revealed the presence of several genes for lypolytic enzymes. Rv1923 gene, a member of Lip family in *M. tuberculosis* demonstrated the least sequence similarity with its counterpart in non-pathogenic strain *M. smegmatis.* The expression of Rv1923 gene (LipD) was not observed in in vitro growing cultures of *M. tuberculosis* H37Ra while an upregulation of transcription of Rv1923 gene was noticed in oxidative conditions. For detailed characterization of LipD enzyme the Rv1923 gene was cloned in pQE30-UA vector and expressed in *E. coli* M15 cells. LipD was purified from inclusion bodies and refolded with nearly 40 % protein yield. The specific activity of enzyme was calculated to be 16 U/mg with pNP-palmitate as a preferred substrate. Kinetic analysis showed K_m 0.645 mM and V_{max} 24.75 U/ml with pNP-palmitate. Ser-102, Asp-342, and His-369, predicted as the members of the catalytic triad, were confirmed by mutagenesis. Mutagenesis studies revealed that catalytic serine residues located in β-lactamase motifs (S-X-X-K) were responsible for lipolytic activity. Secondary structure analysis by CD spectroscopy demonstrated the presence of α helices and β sheets in the canonical structure of LipD. The enzyme was stable up to 50 °C and was active even at pH 6.0. The expression of enzyme under stress conditions and its activity and stability at high temperature and low pH suggested the possible role of LipD in the survival of mycobacterium in macrophage compartment.

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Introduction

Mycobacterium tuberculosis, causative agent of TB, is an intracellular pathogen which infects mononuclear phagocytes, with a remarkable ability to withstand the bactericidal stresses during the infection process and within the phagosomal compartments. The organism evoked suitable stage-specific responses by interfering with the critical macrophage functions [20]. The bacterium has developed intricate strategies to evade killing mechanisms of phagocytes used for microroganism infection [33]. A key to fight this disease is the identification and characterization of all these different strategies [37].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are highly versatile hydrolytic enzymes that catalyze the hydrolysis and synthesis of a variety of acylglycerols at the interface of lipid and water [14]. A large number of lipases were identified and characterized as virulence factor in many pathogenic bacteria such as *Helicobacter pylori*, *Pseudomonas cepacia*, and *Staphylococcus aureus* [16, 25,

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26] and in fungal species like *Alternaria brassicicola*, *Candida albicans*, and *Fusarium graminearum* [2, 34, 36]. Some of these have been reported to be involved in the degradation of host lipids to provide fatty acids to pathogens [3, 7]. Once inside the macrophage, the mycobacterium utilize lipid metabolism for its growth and survival during dormant stage [8, 28, 29]. Several genes are annotated as lipase/esterase in mycobacterium genome sequence [4]. A bioinformatics analysis has revealed the presence of lip family genes (24 putative lipases) in *M. tuberculosis* H37Rv [10]. Till date out of these 24 putative lipases, few have been cloned and characterized [5, 30, 38]. Little is known about the physiological role of these lipolytic enzymes as well as their role in lipid metabolism. Extensive accumulation and degradation of TAGs in the bacilli was observed during entry into and exit from hypoxia-induced dormancy [17]. LipY, a member of lip gene family, was reported to be responsible for the utilization of accumulated triglycerols (TG) during dormancy and reactivation of the pathogen from dormancy [10]. LipF, an acid-inducible lipase, was reported to have a role in pathogenesis of mycobacterium [24, 38] but no long-chain TG hydrolysis was detected with LipF [38]. LipC was identified as cell surface protein that elicited strong humoral immune responses [30]. Therefore, detailed characterization of all putative lipases would be required to support their role in different environmental conditions. A study of lipolytic enzymes in *M. tuberculosis* could open a new approach to drug targeting, where the primary targets are enzymes involved in lipid degradation rather than crucial enzymes involved in lipid biosynthesis [32].

In the present investigation Rv1923 gene product (LipD) from *M. tuberculosis* H37Rv, annotated as an intracellular putative lipolytic enzyme, was selected. Though annotated as a member of lip family, LipD showed similarity with β-lactamase. β-lactamases were partly attributed to their drug resistance to certain β-lactam antibiotics. Therefore, precise biochemical properties and the expression pattern of LipD were urgently needed to be elucidated. Out of all putative lipases, Rv1923 shared the least similarity with its counterpart in non-pathogenic strain *M. smegmatis* while no change in gene-coding sequence was noticed in its attenuated identical *M. tuberculosis* H37Ra strain. Once inside the macrophage, mycobacterium expresses certain genes to circumvent the different stress conditions [11]. These gene products are key to the survival of this intracellular pathogen. Therefore, the expression of Rv1923 gene was checked in in vitro culture conditions of *M. tuberculosis* H37Ra under various growth and stress conditions. The Rv1923 gene was cloned, expressed and the protein was purified for detailed biochemical and biophysical characterization. We hypothesized its role in survival of pathogen inside the macrophage if the enzyme

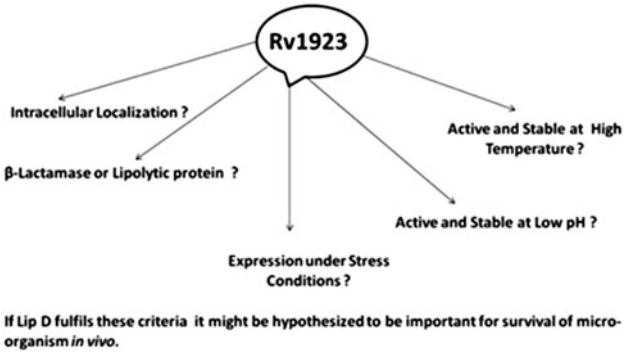

Fig. 1 Hypothesis of Rv1923 to help mycobacterium to survive in vivo

demonstrated activity and stability in acidic conditions and at higher temperature as well as expressed under different stress conditions (Fig. 1.).

Materials and Methods

Databases and Software

The DNA sequence of the Rv1923 gene was retrieved from the NCBI GenBank. BLAST online http://blast.ncbi.nlm.nih.gov was used for analyzing the homologous identity of the Rv1923 of *M. tuberculosis* with other mycobacterial species. The identical protein sequences of the Rv1923 in different species were aligned with Clustal X1.83 http://www.clustal.org/ and all the positions containing gaps were eliminated. Secondary structure was predicted with help of software Phyre (Imperial college, London).

Rv1923 Gene Expression Analysis in *M. tuberculosis* H37Ra in In Vitro Stress Conditions

Mycobacterium tuberculosis H37Ra was grown in middlebrook 7H9 broth base (Himedia) supplemented with 1 % glycerol and 0.05 % Tween-20. An additional 1 % (v/v) growth supplement OADC (BBL) was added. The cells were grown to mid-log phase (A_{600} 0.7–0.8) and stationary phase. The cells were harvested by centrifugation. To study the expression of Rv1923 in different stress condition, the bacterial pellet in mid-log phase was resuspended in different media for exposure to stress conditions such as oxidative stress (Middle brook 7H9 growth media with 5 mM H_2O_2), acidic stress (7H9 growth media at pH 5.0), and nutrient stress (1X phosphate buffer saline). These three stressed cultures were grown for 6 h, along with the control culture in middlebrook 7H9 broth base (normal pH, without any additive).

RNA Isolation and First Strand cDNA Synthesis

Total RNA was isolated from all the samples using TRI-Reagent as per Sigma-Aldrich manual. The concentration of the total RNA was measured by Nano-Drop (Eppendorf, India) and the integrity of the RNA was evaluated by band intensity of 23S and 16S ribosomal RNA on a 1.5 % agarose gel. First strand cDNA was synthesized from 1.5 µg of total cellular RNA in a 20 µl reaction volume using RevertAid first strand cDNA synthesis kit (Fermentas, USA).

Real-Time PCR Analysis

Transcription of Rv1923 gene was 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. 10 µl of master mix containing enzyme and SYBR Green dye, 0.4 µl of ROX dye, 1 µl each of forward and reverse primer, and double-distilled water is added to complete 20 µl reaction mixture. The experiment was set up following PCR program on The LightCycler® 480 (Roche Applied Science). Amplification was performed in triplicate wells for each sample analyzed; 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. Determination of expression level of the gene was carried out by using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ method [15]. Ct values for each run were used to calculate mean Ct with standard deviation (SD). For each sample: ΔCt test = Ct test−Ct 16S rRNA, ΔCt control = Ct control−Ct 16S rRNA. ΔΔCt values relative to control were calculated by following formula: ΔΔCt = ΔCt test−ΔCt control. The fold change in test gene expression was determined by taking 2 to the power of ΔΔCt values i.e., $2^{-\Delta\Delta Ct}$.

Gene Cloning

Mycobacterium tuberculosis H37Rv chromosomal DNA was a kind gift from Dr. Charu Sharma, CDRI Lucknow, India. The coding region of Rv1923 from *M. tuberculosis* H37Rv chromosomal DNA was amplified using the primers GSF1 (5′-TTG GAT GTA GCG GGG CTA CC-3′) and GSR1 (5′-GAG CAG TTA TGC ACC CGA GG-3′). The thermocycler (Techne Progene, Cambridge, UK) was programmed for a hot start at 94 °C for 4 min followed by 30 cycles at 94 °C for 30 s, 62 °C for 40 s, and 72 °C for 2 min and a final cycle of 7 min at 72 °C. The amplified DNA was purified and ligated to the pQE30-UA vector (Qiagen, MD, USA) to generate construct pQE-LipD and used for transformation of *E.coli* M15 cells. The transformants were screened for the presence of Rv1923 gene by colony PCR and sequencing. The transformant was grown in Luria–Bertani (LB) medium with antibiotics ampicillin (100 µg/ml) and kanamycin (30 µg/ml) at 37 °C with shaking.

Expression and Purification of LipD

For expression of recombinant protein, 1 l of Luria broth medium supplemented with kanamycin (30 µg/ml) and ampicillin (100 µg/ml) was inoculated with 10 ml of an overnight grown culture and incubated at 37 °C with shaking. The culture at mid-log phase ($A_{600} \sim 0.6$) was induced with 0.5 mM isopropyl thio-β-D-galactosidase (IPTG) for 3 h at 37 °C. Cells were harvested at 4 °C by centrifugation at 5,000 g for 15 min and the pellet was 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 5 ml/g wet weight and stored overnight at −80 °C. The cell suspension was thawed on ice for 1 h and 20 mM $MgSO_4$ (final concentration) was added. Cells were disrupted by ultrasonication (15 s cycle × 10) using a Pharmacia Biotech Sonifier with intermittent cooling. The cell extract was centrifuged at 14,000 g for 20 min. The supernatant and pellet were separated. LipD was present in pellet. The pellet was solubilized by stirring at 4 °C overnight in a 40 ml solution containing 10 mM TrisCl (pH 8.0), 100 mM sodium dihydrogen phosphate, and 6 M guanidine hydrochloride. The inclusion bodies were separated from solubilized protein by centrifugation at 14,000×*g* for 20 min at 4 °C. Recombinant Rv1923 was purified from solubilized inclusion bodies by metal affinity chromatography under denaturing conditions using a nickel nitrilotriacetic acid (NiNTA) column as described by the manufacturer (Qiagen). Solubilized inclusion bodies were loaded on a NiNTA column previously equilibrated with equilibration buffer (10 mM TrisCl (pH 8.0), 100 mM sodium dihydrogen phosphate, and 8 M urea). The column was washed with equilibration buffer at pH 6.3, and the bound protein was eluted using a pH gradient at pH 6.3–4.3. The fractions were collected. Protein concentration was determined for each fraction by measuring absorbance at A_{280}. The fractions containing high concentration of protein were pooled, refolded, and analyzed on SDS-PAGE.

Refolding of LipD

Refolding was carried out with chaotropic agents' concentration gradient dialysis. The solution of denatured

protein (1.5 mg/ml) was dialyzed stepwise against 2 l of freshly made 6, 4, 2, 1, 0.5, and 0 M urea in 10 mM sodium phosphate buffer (pH 8.0), respectively. With each concentration, the protein was dialyzed 6 h at 4 °C. The protein concentration of dialyzed sample was determined by BCA method. The enzyme activity of the purified refolded sample was determined.

Lipase Assay

The enzyme activity was determined according to the modified method of Sigurgisladottirr et al. [31]. 100 µl enzyme (100 mg/ml) and 0.1 ml 0.002 M pNP-palmitate were added in 0.8 ml of assay buffer [0.05 M phosphate buffer (pH 8.0), 100 mM NaCl, and 2 mM sodium taurodeoxycholate]. The reaction was carried out at 40 °C for 15 min, after which 0.25 ml 0.1 M Na_2CO_3 was added. The mixture was centrifuged and the activity was determined by measuring absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme, which liberates 1 µmole of *p*-nitrophenol from pNP-ester as substrate per min under the standard assay conditions.

Biochemical Properties of Rv1923

All the experiments were carried out independently in triplicates.

Effect of pH and Temperature on Enzyme Activity and Stability

Effect of temperature on enzyme activity was monitored by carrying out the enzyme assay at different temperatures (20–90 °C). Temperature stability of LipD was determined by incubating enzyme at different temperatures (20–90 °C) for 30 min and pH 8.0 followed by cooling in ice and residual enzyme activity was measured at optimum temperature. The enzyme without treatment was used as control (100 %).

Effect of pH on enzyme activity was monitored by carrying out the enzyme assay at 40 °C with buffers of different pH values (pH 2–10.5). The following buffers (50 mM) were used: glycine/hydrochloric acid, pH 2–3; acetate buffer, pH 4.0–5.5; phosphate buffer, pH 6.0–8.0; TrisCl buffer, pH 8.0–9.0; and carbonate bicarbonate buffer, pH 10.0–10.5. The pH stability of the lipase was determined by incubating the enzyme at different pH values for 1 h at room temperature. The residual lipase activity was determined by the standard assay method at pH 8.0. The enzyme without treatment was used as control.

Substrate Specificity

The substrate specificity of the purified recombinant LipD was measured using pNP esters (Sigma) with carbon chain length ranging from C_4 to C_{18}. The released pNP from the substrates *p*-nitrophenyl butyrate (pNPB; C_4), *p*-nitrophenyl octanoate (pNPB; C_8), *p*-nitrophenyl deconoate (pNPD; C_{10}), *p*-nitrophenyl laurate (pNPL; C_{12}), *p*-nitrophenyl myristate (pNPM; C_{14}), *p*-nitrophenyl palmitate (pNPP; C_{16}), and *p*-nitrophenyl stearate (pNPS; C_{18}) was monitored using spectrophotometric assay. The enzyme assay was carried out as per the standard assay conditions.

Enzyme Kinetics

The effect of pNP-palmitate concentration (0.1–5 mM) on the reaction rate of the enzyme was measured by using the standard enzyme assay. The Michaelis–Menten constant (K_m) and maximum velocity for the reaction (V_{max}) were determined by Lineweaver–Burk plot and k_{cat} and k_{cat}/K_m were calculated.

β-Lactamase Assay

The β-lactamase activity was determined according to the modified method of Perez Llaerna et al. [22]. β-Lactamase activity was assessed spectrophotometrically by hydrolysis of nitrocefin. The assay mixture contained 20 µL of 0.5 mg nitrocefin, 10 % glycerol, and 80 µL (0.6 mg/mL of protein) in a final volume of 100 µL along with control containing 80 µL of 50 mM phosphate buffer in place of enzyme. β-Lactamase activity was monitored by appearance of color change from yellow to red as well as measuring the absorbance at 482 nm.

Site-Directed Mutagenesis

To confirm the probable active site residues of this enzyme identified by sequence alignment and structure model, site-directed mutagenesis of Ser-102 (nucleophilic residue), Asp-342 (charge relay network), and His-369 (proton carrier) of Rv1923 protein was carried out. Three mutant proteins were created, each with a single residue of the putative catalytic triad, Ser-102, Asp-342 being replaced with alanine and His-369 being replaced with proline. Mutagenesis of residues was created with the help of site-directed mutagenesis kit (Staratgene) as per manufacturer's protocol (Table 1). The gene sequencing was carried out to confirm the mutation. The mutated Rv1923 was expressed, purified, and refolded along with the wild type. The protein was analyzed on SDS-PAGE. The enzyme activity of the wild type and mutant proteins was determined and compared.

Table 1 Primer sequences used in the study

Primer name	Sequence
t745g	5′-CACCGGCGGGGCCGCCGGTGC-3′
t745g_antisense	5′-GCACCGGCGGCCCCGCCGGTG-3′
a1031c	5′TGTTGCACGGTGAGAAGGCTCCGATGGTGC-3′
a1031c_antisense	5′-GCACCATCGGAGCCTTCTCACCGTGCAACA-3′
a1130c	5′-CCAACGCCCACCCCGCGTTCGACCT-3′
a1130c_antisense	5′-AGGTCGAACGCGGGGTGGGCGTTGG-3′
LipD Fwd 448	5′ CATCGGTCCGGACTGGCG 3′
LipD Rev 894	5′ GTTGACCGCCGGAACCTCA 3′
16S RNA	
Fwd	5′ GAG GAA GGT GGG GAT GAC GT 3′
Rev	5′ AGG CCC GGG AAC GTA TTC AC 3′

Circular Dichroism (CD) Spectroscopy

Far-UV CD spectra were collected at 24 ± 1 °C on a Chirascan (Applied Photophysics) spectrometer calibrated using 10-camphorsulfonic acid. The protein was dialyzed extensively against 10 mM potassium phosphate buffer (pH 8.0) prior to measurements. Spectrum of purified, refolded LipD (protein concentration, 100 µg/ml) in 10 mM phosphate buffer, pH 8.0, was recorded in the far-UV region from 195 to 260 nm using a cuvette with a path length of 2 mm and an averaged over six spectral scans. Following are the instrument parameters: step size, 1 nm; time per point, 2 s; and scan speed, 30 nm/min. Thermal and pH stability was assessed with increasing temperature from 20 °C to 90 °C and pH (2–9) with 15 and 30 min incubation time, respectively. All CD spectra were corrected using buffer baseline and smoothed using Pro-Data software available with the Chirascan CD spectrometer.

Results

In Silico Studies

When compared with homologous sequences of other mycobacterial species, the Rv1923 sequence showed the least identity to β-lactamase of non-virulent strain, *M. smegmatis* (Table 2). The protein is rich in hydrophobic amino acids (28 %). It has 50.27 % negatively charged amino acids and 28.06 % positively charged amino acids. No transmembrane helices were identified by TMHMM software. Signal P analysis predicted signal sequence in the LipD protein. However, the signal sequence was reported to remain uncleaved [9]. Therefore, it is expected to be present as either membrane-bound protein or intracellular fractions. There is only one cysteine in the protein sequence ruling out the possibility of disulfide bond. Interestingly, LipD demonstrated very low identity with known hydrolases of few bacterial species such as *Arthrobacter nitroguajacolicus* Rue61a (PDB: 3ZYT_A, 31 % identity), *Ochrobactrum anthropi* Sv3 (PDB: 2DNS_A, 24 % identity), and *Burkholderia gladioli* (PDB: 1CI9_A, 34 % identity). BLASTP analysis revealed that LipD fall into class C β-lactamases belonging to the family of VIII carboxylesterases [1]. Sequence alignment of the protein sequence closely related to LipD with respect to conserved catalytic site residues was carried out by ESPript (http://espript.ibcp.fr/ESPript/ESPript/) [13]. Ser-102, His-369, and Asp-342 were conserved and predicted to be active site residues (Supplementary Fig. 1). Like other common lipases, the LipD do not have characteristic lipase/esterase pentapeptide motifs (G-X-S-X-G). The catalytic serine residue was located in β-lactamase motifs (S-X-X-K). A histidine residues involved in catalysis was located in HIG box, another characteristic feature of lipolytic enzyme resembling class C β-lactamases [5]. The enzyme was structurally similar to that of carboxylesterases which showed activity toward short carbon chain esters. The protein structure of LipD was predicted by homology modeling, using online software—Phyre with esterase, EstB from *B. gladioli* as a template (estimated precision-100 %) and image was produced and labeled by PyMol. The predicted model exhibited the typical α/β hydrolase fold, which is characteristic of lipases. Serine residue was found to be buried in the hydrophobic core with the presence of a region of non-polar residues in the form of a lid (Supplementary Fig 2a, b).

Expression Analysis of *Rv1923*

The expression level of transcript was analyzed by real-time PCR. No expression of Rv1923 was observed in normal in vitro growing *M. tuberculosis* H37Ra in log phase or stationary phase. However, there was upregulation

Table 2 Percentage homology of Rv1923 nucleotide sequence with other species of Mycobacterium by using BLAST

Gene/gene product	*M. smegmatis*	*M. marinum*	*M. leprae*	*M. abscessus*	*M. bovis*
Rv1923/LipD	69 % with β-lactamase	80 % with LipD	64 % with esterase	68 % with putative lipase/ β-lactamase	99 % with putative lipase, LipD

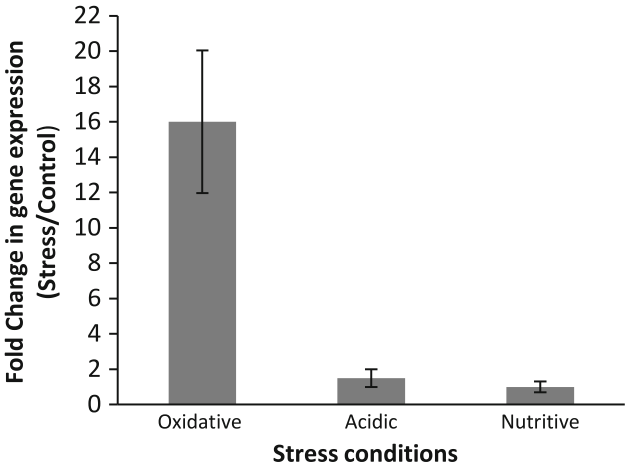

Fig. 2 Expression analysis of regulated gene Rv1923 in different stress conditions by real-time RT-PCR. Levels of fold change of mRNA expression of Rv1923 (fold change) were calculated along with internal control (16S rRNA). Experiments were done in triplicate ($n = 3$) and *error bars* indicate standard deviation among the triplicate samples

of expression of *Rv1923* in all the three stress conditions. But the enhancement was not significant in acidic and nutritive stress. Nearby 16-fold enhanced expression was observed in oxidative stress (Fig. 2). 16S RNA was used as an internal control as used in all the bacterial expression studies.

Cloning of Rv1923 Gene

Rv1923 gene of *M. tuberculosis* H37Rv, with its own start codon was amplified using chromosomal DNA as template. The amplified product corresponded with the expected size (1,341 bp) of Rv1923 of *M. tuberculosis* H37Rv. Transformants were screened by colony PCR for the presence of Rv1923 insert. The amplified product of approximately the same size (1.3 kbp) confirmed the presence of insert. The sequencing results showed 100 % similarity with Rv1923 nucleotide sequence in *M. tuberculosis* H37Rv genome [6].

Purification of LipD

We could not express LipD in soluble form. The protein was recovered from inclusion bodies. After solubilizing in GndHCL, the denatured enzyme was purified to homogeneity

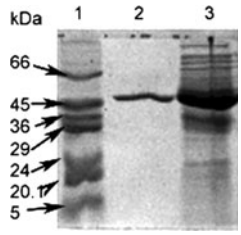

Fig. 3 SDS-PAGE analysis of purified LipD. *Lane 3* lysate, *Lane 2* purified LipD, and *Lane 1* protein molecular weight marker

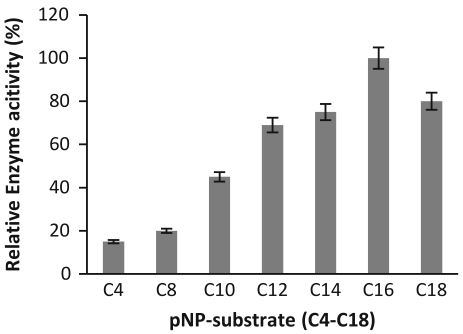

Fig. 4 Substrate specificity of recombinant LipD. The substrate specificity of the purified recombinant LipD was measured using pNP-esters (Sigma) with carbon chain length ranging from C_4 to C_{18}. The reaction mix without enzyme served as blank. Values are the average results from three independent assays in triplicates, with *error bars* indicating SD

by affinity chromatography on Ni–NTA column as a His-tagged protein. The enzyme was refolded to nearly 40 % protein yield with specific activity of 16 U/mg protein. The protein migrated as a single band on denaturing SDS-PAGE with an approximate molecular mass of 48 kDa (Fig. 3, lane 2). The molecular mass was little higher than the calculated mass of total amino acid residues as the protein was expressed as fusion protein.

Substrate Specificity

Maximum hydrolytic activity was observed with pNP-palmitate (C_{16}) substrate. A decrease in enzyme activity was noticed as the length of the fatty acid chain decreased and the activity of LipD for pNP-caprate (C_{10}) was only 20 % of that for pNP-palmitate (Fig. 4). These results indicate that LipD can hydrolyze a wide range of pNP-ester substrates with a preference for long-chain fatty acids.

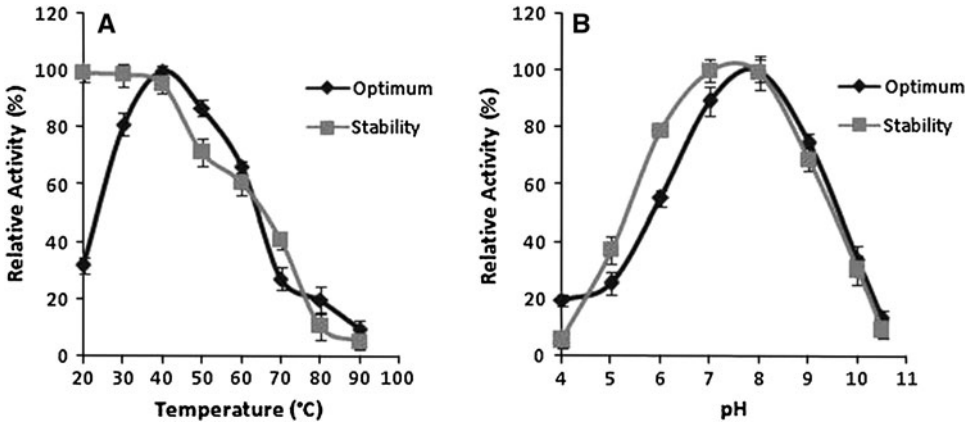

Fig. 5 Temperature (**a**) and pH (**b**) dependence activity and stability of LipD. Values are the average results from three independent assays in triplicates, with *error bars* indicating SD

Effect of Temperature and pH on LipD Activity and Stability

The effect of temperature and pH on the activity of LipD was determined using pNP-palmitate as the preferred substrate. The enzyme demonstrated optimum activity at 40 °C. Although a high level of residual activity (~60 %) was observed at 60 °C, the enzyme could retain 100 % activity up to 40 °C for 30 min (Fig. 5a). The enzyme shows maximal activity at pH 8.0, maintaining an activity above 60 % in the pH range 7.0–9.0 (Fig. 5b). The enzyme was stable for 30 min between pH 6.0 and 9.0 and retained more than 70 % of enzyme activity. It could retain approximately 35 % of enzyme activity after incubation at pH 5.0 for 30 min.

Enzyme Kinetics

Enzyme activity as function of substrate concentration (0.01–5 mM) was determined for LipD. It exhibited a specific activity of 16.1 U/mg of protein. The apparent K_m and V_{max} values for substrate, pNP-palmitate were 0.645 mM and 24.75 U/ml, respectively. The turnover number (k_{cat}) of the enzyme was calculated to be 1.267 min^{-1}.

Active Site Identification of Rv1923

The active site residues of Rv1923 were confirmed by site-directed mutagenesis. The wild type and mutant proteins migrated at the same position in SDS-PAGE (Fig. not shown). For the proteins with altered serine and histidine residues, the enzyme activity was completely lost. Nearly 75 % loss of enzyme activity was observed for Asp-342 mutant in comparison to control.

CD-Spectroscopic Analysis

The observed CD spectrum of native protein was characteristic of α-helical/β-sheet protein (Fig. 6a). Single scan of LipD at far-UV CD spectrum revealed that protein is in refolded state and having both α-helical and β-sheet content in secondary structure at 25 °C (Fig. 6a). To further get insight into the structure stability of the protein, pH-dependent unfolding of the protein secondary structure was monitored (Fig 6b). Variation in pH from 9.0 to 5.0 showed no significant effect on the secondary structure content of the enzyme while lowering the pH below 5, we observed distortion in the structure with complete loss of structure at pH 2.0 (Fig. 6b). When protein was subjected to temperature variation from 20 to 90 °C for thermal unfolding studies, we observed a gradual decrease in negative ellipticity at 208 and 222 nm up to 80 °C. After that, the protein secondary structure was observed to be completely distorted (Fig. 6c). The loss in ellipticity can also be monitored by monitoring changes in CD ellipticity at a fixed wavelength of 222 nm (Fig. 6d). These studies indicated that the structure of LipD was proportionally diverted toward denaturation and complete denaturation was observed after 80 °C (Fig. 6d).

Discussion

Amidst the robust host immune responses, an advantageous survival strategy deployed by the bacterium was to express different sets of proteins to meet the requirements of chronic persistence and also to successfully counteract the host immune attack [20]. As the bacteria grown in vivo preferred fatty acids as their carbon source the lipolytic enzymes were very likely to participate in lipid metabolism during active bacterial growth or stress conditions [24]. In the present investigation, attempt had been made to study

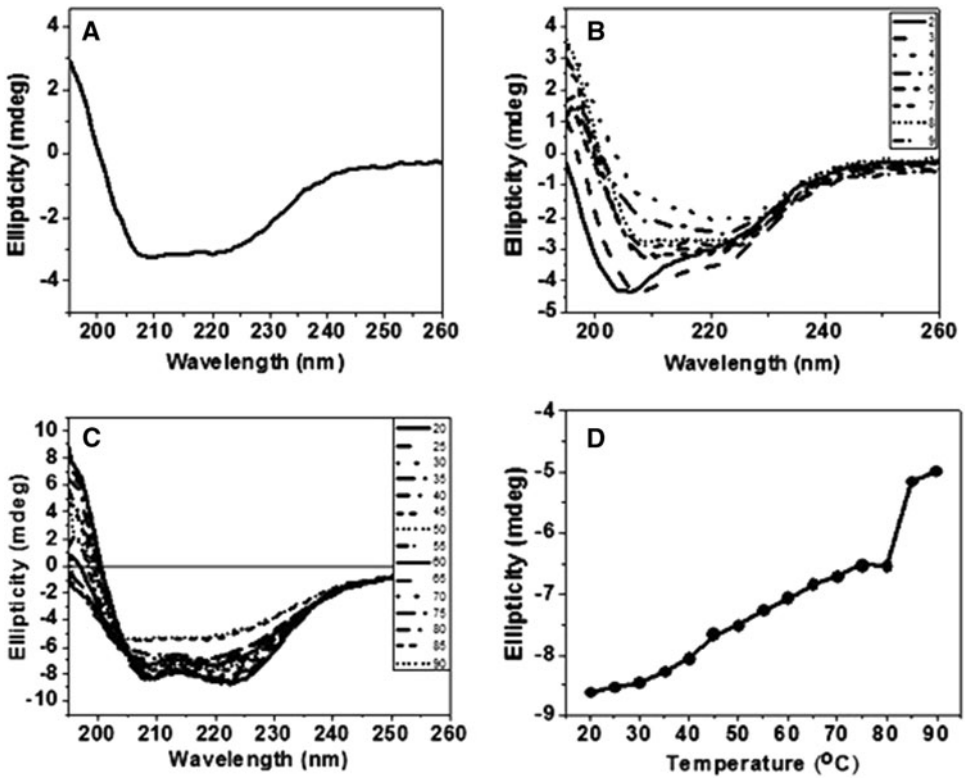

Fig. 6 **a** Far-UV CD spectrum analyses of LipD, **b** CD spectrum analyses of LipD after 30-min incubation at pH 2–9, **c** 15-min incubation at temperature 20–90 °C, **d** temperature melting curve of native protein at 222 nm. The experiment was carried out three times

the expression pattern of Rv1923 gene in different growth and stress conditions and characterize LipD.

Adaptation under different stress conditions appeared to be the key to survival of this intracellular pathogen. The expression of several proteins had been reported to be regulated by several types of stresses (i.e., oxidative, acidic, nutritive, nitrosative, and hypoxic) and consequent induction of non-replicating persistence (NRP) by these stressors had also been reported [19]. Rv1923 demonstrated no expression in the various growth conditions but showed expression in all the three stress conditions with significant expression in oxidative stress indicating its role in in vivo conditions where the mycobacterium is under the multiple stresses together. So far, out of 24 lipases, only LipF is found to be upregulated in acidic stress while no effect was observed in other stress conditions [24].

Maximum hydrolytic activity of LipD was observed with pNP-palmitate as a substrate. Hydrolysis of long carbon chain substrates by LipD suggested that the enzyme could be classified as a lipase. It was earlier reported that the pH of the macrophage compartment, in which *M. tuberculosis* resides, ranges from pH 6.2 to 4.5, depending on the activation state of the macrophage [18, 27, 35]. On the other hand, during initial infection, the internal temperature of the host would be slightly higher than healthy subjects owing to the immune response against the pathogen [12]. In such a condition, temperature- and pH-stable proteins could be predicted to play an essential role for the survival of pathogen [12]. The change in structural stability and functional activity of the LipD complement each other both with respect to pH and temperature. Activity of LipD at 45–50 °C and significant activity in acidic pH range suggested that protein is functionally active at these conditions. The CD spectra results demonstrated that the enzyme was able to retain its secondary structure at high temperature and in acidic conditions. LipD protein carries a putative catalytic domain containing a triad of Ser, Asp and His, residues. The domain is characteristic for α/β hydrolase fold [21], and mutating the catalytic triad residues resulted in total loss of lipase activity. The LipD was expected to show β-lactamase activity also. So further investigation of LipD protein with respect to the ability to hydrolyze β-lactam substrates nitrocefin revealed that this protein has no β-lactamase activity (Data not shown). Similarly EstB belonging to class C β-lactamase of family VIII carboxylesterases demonstrated no β-lactamase activity [23]. The predicted localization of enzyme, its expression in oxidative stress, active and stable at high temperature and low pH suggested its possible role in the survival of microorganism in macrophages.

Conclusion

It was hypothesized that the enzyme LipD, product of Rv1923 gene, might contribute to the survival of mycobacteria in vivo condition. The hypothesis was supported by the fact that LipD has a preference for long carbon chain substrate, pNP-palmitate, as well as activity and stability at low pH and high temperature. Also, the enhanced expression of Rv1923 during the stress conditions forms a strong ground for emphasizing its role in survival of mycobacterium in vivo.

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References

1. Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. Biochem J 343(Pt 1):177–183
2. Berto P, Commenil P, Belingheri L, Dehorter B (1999) Occurrence of a lipase in spores of *Alternaria brassicicola* with a crucial role in the infection of cauliflower leaves. FEMS Microbiol Lett 180:183–189
3. Camacho R, Ensergueix D, Perez E, Gicquel B, Guilhot C (1999) Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Mol Microbiol 34:257–267
4. Canaan S, Maurin D, Chahinian H, Pouilly B, Durousseau C, Frassinetti F, Scappuccini-Calvo L, Cambillau C, Bourne Y (2004) Expression and characterization of the protein Rv1399c from *Mycobacterium tuberculosis*. A novel carboxyl esterase structurally related to the HSL family. Eur J Biochem 271:3953–3961
5. Cha SS, An YJ, Jeong CS, Kim MK, Jeon JH, Lee CM, Lee HS, Kang SG, Lee JH (2013) Structural basis for the β-lactamase activity of EstU1, a family VIII carboxylesterase. Proteins. doi:10.1002/prot.24334
6. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537–544
7. Cotes K, Dhouib R, Douchet I, Chahinian H, de Caro A, Carrière F, Canaan S (2007) Characterization of an exported monoglyceride lipase from *Mycobacterium tuberculosis* possibly involved in the metabolism of host cell membrane lipids. Biochem J 408:417–427
8. Daniel J, Maamar H, Deb C, Sirakova TD, Kolattukudy PE (2011) *Mycobacterium tuberculosis* uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. PLoS Pathog 7(6):e1002093. doi:10.1371/journal.ppat.1002093
9. de Souza GA, Leversen NA, Målen H, Wiker HG (2011) Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. J Proteomics 21(75):502–510
10. Deb C, Daniel J, Sirakova TD, Abomoelak B, Dubey VS, Kolattukudy PE (2006) A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. J Biol Chem 281:3866–3875
11. Fontán P, Aris V, Ghanny S, Soteropoulos P, Smith I (2008) Global transcriptional profile of *Mycobacterium tuberculosis* during THP human macrophage infection. Infect Immun 76(2):717–725
12. Ganaie AA, Lella RK, Solanki R, Sharma C (2011) Thermostable hexameric form of eis (Rv2416c) protein of *M. tuberculosis* plays an important role for enhanced intracellular survival within macrophages. PLoS One 6(11):e27590
13. Gouet P, Courcelle E, Stuart DI, Metoz F (1999) ESPript: multiple sequence alignments in PostScript. Bioinformatics 15:305–308
14. Jaeger KE, Ransac S, Dijkstra BW, Colson C, van Heuvel M, Misset O (1994) Bacterial lipases. FEMS Microbiol Rev 15:29–63
15. Livak KJ, Schmittgen TD (2005) Analysis of relative gene expression data using realtime quantitative PCR and the 2ΔΔC (T) method. Methods 25:402–408
16. Lonon MK, Woods DE, Straus DC (1998) Production of lipase by clinical isolates of *Pseudomonas cepacia*. J Clin Microbiol 26:979–984
17. Low KL, Rao PS, Shui G, Bendt AK, Pethe K, Thomas D, Wenk MR (2009) Triacylglycerolutilization is required for regrowth of in vitro hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. J Bacteriol 191:5037–5043
18. MacMicking JD, Taylor GA, McKinney JD (2003) Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science 302:654–659
19. Manganelli R, Proveddi R, Rodrigue S, Beaucher J, Gaudreau L, Smith I (2004) Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. J Bacteriol 186:895–902
20. Narayana Y, Joshi B, Katoch VM, Mishra KC, Balaji KN (2007) Differential B-cell responses are induced by *Mycobacterium tuberculosis* PE antigens Rv1169c, Rv0978c, and Rv1818c. Clin Vaccine Immunol 14(10):1334–1341
21. Nardini M, Dijkstra BW (1999) Alpha/beta hydrolase fold enzymes: the family keeps growing. Curr Opin Struct Biol 9(6):732–737
22. Perez-Llarena F, Martín JF, Galleni M, Coque JJ, Fuente JL, Frère JM, Liras P (1997) The bla gene of the cephamycin cluster of Streptomyces clavuligerus encodes a class A beta-lactamase of low enzymatic activity. J Bacteriol 179(19):6035–6040
23. Petersen EI, Valinger G, Solkner B, Stubenrauch G, Schwab H (2001) A novel esterase from *Burkholderia gladioli* which shows high deacetylation activity on cephalosporins is related to beta-lactamases and DD-peptidases. J Biotechnol 89(1):11–25
24. Richter L, Saviola B (2009) The lipF promoter of *Mycobacterium tuberculosis* is upregulated specifically by acidic pH but not by other stress conditions. Microbiol Res 164:228–232
25. Rollof J, Braconier JH, Soderstrom C, Ehle PN (1988) Interference of *Staphylococcus aureus* lipase with human granulocyte function. Euro J Clin Microbiol Infect Dis 7:505–510
26. Ruiz C, Falcocchio S, Pastor FI, Saso L, Diaz P (2007) Helicobacter pylori EstV: identification, cloning, and characterization of the first lipase isolated from an Epsilon-proteobacterium down-pointing small open triangle. Appl Environ Microbiol 73(8):2423–2431
27. Schaible UE, Sturgill-Koszycki S, Schlesinger PH, Russell DG (1998) Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium* containing phagosomes in murine macrophages. J Immunol 160:1290–1296

28. Segal W, Bloch H (1956) Biochemical differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. J Bacteriol 72:132–141
29. Segal W, Bloch H (1957) Pathogenic and immunogenic differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. Am Rev Tuberc Pulm Dis 75:495–500
30. Shen G, Singh K, Chandra D, Serveau-Avesque C, Maurin D, Canaan S, Singla R, Behera D, Laala S (2011) LipC (Rv0220) is an immunogenic cell surface esterase of *Mycobacterium tuberculosis*. Infect Immun 80:243–253
31. Sigurgisladottirr S, Konraosdottir M, Jonson A (1993) Lipase activity of thermophilic bacteria from Icelandic hot spring. Biotechnol Lett 15:361–366
32. Singh G, Singh G, Jadeja D, Kaur J (2010) Lipid hydrolizing enzymes in virulence: *Mycobacterium tuberculosis* as a model system. Crit Rev Microbiol 36:259–269
33. Ting LM, Kim AC, Cattamanchi A, Ernst JD (1999) *Mycobacterium tuberculosis* inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 163:3898–3906
34. Tsuboi R, Komatsuzaki H, Ogawa H (1996) Induction of an extracellular esterase from *Candida albicans* and some of its properties. Infect Immun 64:2936–2940
35. Via LE, Fratti RA, McFalone M, Pagan-Ramos E, Deretic D, Deretic V (1998) Effects of cytokines on mycobacterial phagosome maturation. J Cell Sci 111:897–905
36. Voigt CA, Schäfer W, Salomon S (2005) A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. Plant J 42:364–375
37. Wilson JW, Schurr MJ, LeBlanc CL, Ramamurthy R, Buchanan KL, Nickerson CA (2002) Mechanisms of bacterial pathogenicity. Postgrad Med J 78:216–224
38. Zhang M, Wang JD, Li ZF, Xie J, Yang YP, Zhong Y, Wang HH (2005) Expression and characterization of the carboxyl esterase Rv3487c from *Mycobacterium tuberculosis*. Protein Expr Purif 42:59–66