Mycobacterium tuberculosis LipE has a lipase/esterase activity and is important for intracellular growth and in vivo infection.

Running Title: Mtb LipE is important for intracellular growth

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ABSTRACT

*Mycobacterium tuberculosis* (Mtb) Rv3775 (LipE) was annotated as a putative lipase. However, its lipase activity has never been characterized and its precise role in tuberculosis (TB) pathogenesis has not been thoroughly studied to date. We overexpressed and purified the rLipE protein and demonstrated that LipE has a lipase/esterase activity. rLipE prefers medium-chain ester substrates, with the maximal activity on hexanoate. Its activity is the highest at 40 °C and pH=9. We determined that rLipE hydrolyzes trioctanoate. Using site-directed mutagenesis, we confirmed that the predicted putative activity triad residues Ser<sup>97</sup>, Gly<sup>342</sup>, and His<sup>363</sup> are essential for the lipase activity of rLipE. Expression of *lipE* gene was induced under stressed conditions mimicking Mtb’s intracellular niche. The gene-disrupting mutation of *lipE* led to significantly reduced bacterial growth inside THP-1 cells and human peripheral blood mononuclear cell derived macrophages, and attenuated Mtb infection in mice (with ~8-fold bacterial load reduction in mouse lungs). Our data suggest that LipE functions as a lipase and is important for Mtb intracellular growth and *in vivo* infection.
Emerging literature has discovered that Mycobacterium tuberculosis (Mtb) utilizes lipids and fatty acids (FAs) as important nutrients during infection. After phagocytosed by the alveolar macrophages, Mtb can manipulate macrophage to accumulate lipid bodies and form a foamy phenotype (1). During infection, Mtb relies on its lipases to hydrolyze host lipids to release FAs by catalyzing the hydrolysis of ester bonds in long-chain acylglycerols (2, 3). Genomic sequencing of Mtb H37Rv and CDC1551 strains predicted that Mtb possesses more than 250 genes related to lipid metabolism (4, 5). Among them, 24 lipid/ester hydrolases of Mtb were annotated belonging to the “Lip” family (Lip C to Z) (4, 5). Some of the Lip family lipase/esterase activities have been characterized (6-16). Six additional hypothetical genes of Mtb encoding esterases have also been identified, and they contain the pentapeptide motif “GxSxG” shared by most of the Lip family proteins (17). Some proteins involved in lipid metabolism of Mtb are also virulence-related, and mutations of them lead to attenuated phenotypes in cell and animal infection. These include mycolic acids synthases (18), trehalose synthases (19), polyketide synthases (20), FA-CoA synthases (21), isocitrate lyases (22), phospholipases (23), acyl-CoA dehydrogenases (24), lipid carriers (24), and lipid transporters (25-28). Some lipases also play critical roles in Mtb virulence. For example, the gene-disrupting mutation of lipF caused bacterial load reduction in lungs of mice (29, 30). Mutation of another lipase/esterase, Rv2224c, also caused decreased bacterial load in mice (10). Overexpression of LipY in M. bovis Bacillus Calmette-Guérin impaired immune protection against infection in mice (14). Because lipid/ester catabolism is an important requirement for Mtb infection and persistence in hosts, functional characterization of the specific lipases/esterases in Mtb lipid/ester catabolism pathways provides an opportunity to discover new mechanisms of TB pathogenesis.
Dutta et al. used a pool of 326 mutants of Mtb to infect a nonhuman primate model and identified mutants in 108 Mtb genes that were attenuated for \textit{in vivo} growth \cite{31}. LipE was listed as one of them. However, the precise role of LipE in TB pathogenesis has not been thoroughly studied to date. Although the lipase activities of a few of Lip family lipases have been characterized, the activity and function of LipE in Mtb lipid catabolism remain unexplored. In this study, we characterized the lipase/esterase activity of rLipE, determined its catalytic triad and its hydrolysis of triglycerides. We also evaluated its transcriptional expression under stressed conditions that mimic Mtb intracellular niche in phagosome. Finally, we defined the impact of LipE on Mtb intracellular growth in macrophages and its impact on Mtb \textit{in vivo} infection.

\textbf{RESULTS:}

1. \textbf{Amino acid sequence analysis of Rv3775 (LipE) and homology 3D model of LipE.} We obtained the amino acid sequence of Rv3775 (415 AA, 45.3kD) from Tuberculist, in which Rv3775 is annotated as LipE and predicted to belong to the Lip family lipases. We constructed a phylogenetic tree of the 24 Mtb Lip family proteins. It illustrated that LipE might be evolutionarily close to LipD, LipL and LipP (Fig. S1). We then aligned the amino acid sequence of LipE with the sequences of LipD, LipL and LipP and EstA of \textit{Caulobacter crescentus}. The EstA from \textit{C. crescentus} was the template for constructing the homology 3D model of LipE as described below. The sequence alignment revealed that LipE has a SxxK motif at AA 97-100, which is conserved in LipD, LipL, LipP and EstA of \textit{C. crescentus} (Fig. 1A). Homologous known crystal structures that displayed the maximum query coverage and sequence identity with LipE were chosen as templates for generating the 3D model structures in SWISS-MODEL workspace. Three model structures were generated based on homology to other bacterial lipases. They were further evaluated and ranked by measuring the distance between the catalytic residues.
and structural alignment with the templates using PyMol. The 3D model that had the lowest RMSD value and hydrogen bond distance of the catalytic residues between the predicted model and the template was selected as the final model. The results showed that the EstA (PDB: 5gkv.1) from *C. crescentus* had the highest similarity with the LipE sequence (41 identical AA in range 26-405), and the 3D structure model of LipE using the EstA as a template had the lowest RMSD value (RMSD = 0.144). This predicted 3D structure of LipE is composed of seven β-strands, ten α-helices, and six 3_10 helices (Fig. 1B). Combining the protein sequence alignment result with the 3D structure model, we predicted Ser^{97}, Gly^{342}, and His^{363} residues as the putative active triad for LipE (Fig. 1C).

2. **Biochemical characterization of LipE and determination of its catalytic triad.** We overexpressed LipE as a His-tagged fusion protein in *M. smegmatis*. The samples were analyzed by SDS-PAGE, and the results showed that the purified rLipE has a molecular mass of ~45 kD as predicted based on its amino acid sequence (Fig. 2A). The enzymatic assay of rLipE was performed using various *p*-NP-ester substrates, with the *p*-NP-esters in the absence of rLipE as the negative controls. We found that rLipE preferentially hydrolyzed medium-chain *p*-NP-esters: *p*-NP-C6, -C8, and -C10, had low activities with long-size *p*-NP-C12 and -C14, and had no activities with *p*-NP-C2, -C4, -C16, and -C18 (Fig. 2B). Its optimal temperature was 40 °C (Fig. 2C). rLipE had high activity in a wide range of pH 7-11, with the optimal pH = 9, but no activity at pH=6 or lower (Fig. 2D). We determined its dynamic parameters with the *p*-NP-C6 as follows: specific activity= 560.95±16.61 U/mg; $K_m$ = 711.58±54.63 µM; $V_{max}$ = 6756.76±717.49 mM/min, using Lineweaver-Burk plot (Fig. 2E & F). Our phylogenetic analysis indicates that LipE is evolutionarily close to LipD and LipL. The active triad residues in LipD were reported as Ser^{102}, Asp^{342}, and His^{369} (13), whereas in LipL the key catalytic amino acid residues were identified as
Based on our protein sequence alignment result and the 3D model of LipE, we predicted Ser\textsuperscript{97}, Gly\textsuperscript{342}, and His\textsuperscript{363} residues as the putative active triad for LipE (Fig. 1C & E). We conducted site-directed mutagenesis to generate single amino acid substitution mutations for the predicted active site residues Ser\textsuperscript{97}, Gly\textsuperscript{342}, and His\textsuperscript{363} to Ala, and extracted the mutant rLipE proteins (Fig. S2). Because Ser\textsuperscript{97}, Lys\textsuperscript{100}, and His\textsuperscript{363} are predicted as catalytic residues of LipE for its β-lactamase activity (shown below), we also constructed a K100A mutant rLipE protein to investigate its impact on the lipase activity of LipE. G342A, and K100A mutations led to the enzymatic activities of LipE reduced by >95%, and the S97A and H363A mutations completely demolished the lipase activity of LipE, indicating that Ser\textsuperscript{97}, Lys\textsuperscript{100}, Gly\textsuperscript{342}, and His\textsuperscript{363} are essential for LipE’s lipase activity (Fig. 2D).

3. LipE is capable of hydrolyzing triglycerides. We determined activity of LipE on triglyceride hydrolysis using TLC with trioctanoate as a substrate. The monoctanoate and dioctanoate were used as standards in this assay. The results of TLC revealed that rLipE can hydrolyze trioctanoate into dioctanoate (Fig. 3), implying that LipE possesses triacylglycerol acyl hydrolase activity. The three mutant rLipEs (S97A, G342A, and H363A) cannot hydrolyze trioctanoate, indicating these three residues are essential for the activity of triacylglycerol hydrolysis.

4. LipE hydrolyzes fluorocillin green, a β-lactamase substrate. We investigated homology of LipE amino acid sequence using Protein-BLAST and found that it has homologies to class A β-lactamase-related serine hydrolase. We determined its β-lactamase activity using a fluorogenic β-lactamase substrate, fluorocillin green, which has two attached cephalosporin moieties. The fluorocillin green sample without rLipE served as the negative control to normalize the signal of samples with rLipE. The results demonstrated that rLipE can hydrolyze fluorocillin green with a...
dose-response effect, indicating that it can cleave the cephalosporin ring structure in fluorocillin green (Fig. 4A). We determined its $K_{cat}$ on fluorocillin green as $7.7685 \pm 0.4527 \text{ S}^{-1}$. We then analyzed its amino acid sequence and identified three motif SxxK (97SAAK100, 131SHGK134, and 385SSGK388), and one motif HLG (363HLG365) conserved in β-lactamases. Combining this information with the 3D model, we predicted the catalytic residues of LipE for its β-lactamase activity as Ser$^{97}$, Lys$^{100}$ and His$^{363}$. We verified this hypothesis using the S97A, K100A, and H363A mutant rLipE proteins. As Gly$^{342}$ is predicted to be one of the catalytic residues for LipE’s lipase activity, we also examined its impact on LipE’s β-lactamase activity. The S97A and K100A mutations completely demolished rLipE’s β-lactamase activity; whereas the mutation of H363A kept less than 10% activity (Fig. 4B). The G342A mutation reduced rLipE’s β-lactamase activity to the level of <2%. These data suggest that Ser$^{97}$ and Lys$^{100}$ are absolutely required for its β-lactamase activity, and Gly$^{342}$ and His$^{363}$ are also important for its β-lactamase activity. We further determined susceptibility of the LipE mutant strain to β-lactam antibiotics using the Resazurin Microtiter Assay (REMA). Compared to the MICs of the tested β-lactam antibiotics against the wild-type and lipE complemented strains, the lipE::Tn mutation only led to a 2 - 4 fold MIC reduction, suggesting that LipE may not play a major role in resistance to β-lactam antibiotics (Table 1).

5. Expression of lipE gene is induced under stressed conditions. Mtb inside macrophages is exposed to several stressed conditions, such as reactive nitrogen intermediates and reactive oxygen species. When the phagosomes mature and sequentially fuse with lysosomes, Mtb faces the highly acidic and hydrolytic milieu of lysosomes (33). We studied lipE gene expression under the following four stressed conditions that mimic Mtb intracellular niche: 1) oxidative stress (5 mM H$_2$O$_2$ in the regular medium at 37 °C); 2) acidic stress (regular medium with...
pH=4.5 at 37 °C; 3) nutritive stress (1XPBS buffer at 37 °C); and 4) heat stress (regular medium at 40 °C). Bacterial RNA was extracted at 15 min or 6-h after inoculation. The rv3203 (lipV) gene of Mtb, which encodes another Lip family lipase, was reported to be only induced under the acidic stress when tested under these four conditions, so it served as a control (34). 16S-rRNA was used as an internal control to normalize the expression level under the stressed or normal conditions. The gene expression ratio was calculated as the normalized transcript level under the stressed condition divided by the normalized transcript level under the normal culture condition (Fig. 5). Under the nutritive and heat stresses, lipE expression was transiently induced (9.39±0.36 -fold; P<0.0001 and 2.39±0.24 -fold; P<0.0001, respectively), suggesting that LipE was upregulated during the initial adaptation to these two stresses. Under the acidic stress condition, lipE expression was induced at both the early (1.84±0.03 -fold, P<0.0001) and the late (1.75±0.07 -fold, P<0.0001) time points. Upregulation of lipE expression under these stressed conditions indicates that LipE might have a specific role in survival of one or more of these stressed conditions.

6. **Mutation of lipE leads to attenuated intracellular Mtb growth.** To determine the role of LipE in Mtb infection, we acquired a lipE transposon insertion mutant strain (point of insertion at 320 bp of the total 1,248 bp lipE gene) from the BEI Resources. This strain was originally constructed by Dr. William Bishai’s laboratory (35). We then constructed the lipE complemented strain with this strain. The wild type, mutant, and complemented strains were labeled with the tdTomato fluorescent protein by transforming a tdTomato-expressing plasmid (36). We studied the impact of lipE::Tn mutation on Mtb growth in a common liquid medium, MOAD-Tw, and found that there were not significant differences in growth among the wild-type, mutant, and complemented strains (Fig. S3). We compared cell invasion rates between the wild-type, the
lipE::Tn mutant, and the complemented strains using the THP-1 cell line. We did not observe significant differences in cell invasion rates among these three strains (Fig. S4). We then studied the impact of lipE mutation on Mtb intracellular growth in infected macrophages. These strains were applied to infect PMA stimulated THP-1 cell line and human PBMC derived macrophages in 96-well plates. The tdTomato specific fluorescence intensity (FI) was measured at 0-h after removing extracellular bacteria as the baseline fluorescence, and then measured daily for four days. The intracellular growth ratio of each strain was calculated to normalize the variation of initial bacterial numbers entering macrophage (Fig. 6A). Cells were lysed at day-4 after reading fluorescence. The intracellular Mtb was titrated and plated on 7H11 agar medium for CFU enumeration (Fig. 6B). In THP-1 cells, the intracellular growth of lipE mutant strain was significantly lower than the wild-type and complemented strains. In human PBMC derived macrophages, the lipE mutant strain also showed significantly lower intracellular growth than the wild-type and complemented strains in both the fluorescence-based growth ratio and CFU data at day-4.

7. **Mutation of lipE leads to reduced bacterial load in lungs of infected mice.** The cell infection results suggest that LipE is important for Mtb intracellular survival. We selected C3HeB/FeJ mice as an animal model for Mtb aerosol infection to determine LipE’s impact on Mtb in vivo infection, because this mouse model develops highly organized granulomas with centered necrotic lesions in the lungs resembling human pulmonary lesions (37). We compared bacterial load in the lungs of mice at day-14, -28, and -56 post infection. The mutation of lipE led to a significant bacterial load reduction by CFU enumeration compared to the wild-type and complemented strains at day-28 and -56 post infection (Fig. 7A). In consistent with the CFU data, IVIS imaging of the lung tissues from the mice of the three groups showed that the mutant strain
infected mice had significantly lower fluorescence signal from the lungs (Fig. 7B & C). Together, these results suggest that LipE is important for Mtb intracellular survival and in vivo infection.

**DISCUSSION:**

Our cell and mouse infection data strongly support that LipE is important for Mtb survival/growth in macrophages and in hosts. In addition, our results demonstrate that LipE is upregulated by a nutrient-starvation condition. Similarly, another paper also reported that lipE was upregulated by a condition that first induced triglycerol accumulation in Mtb after a 12-day hypoxic growth and then exposed Mtb to starvation (PBS) (8). Furthermore, the TLC data from our study demonstrate that rLipE can hydrolyze glyceryl trioctanoate, a triglycerol with medium-carbon chain. Together, these data highly suggest that LipE might be involved in triglycerol metabolism in Mtb and contribute to Mtb intracellular survival. Several Mtb lipases have been reported to play critical roles in Mtb virulence. For example, mutation of lipF caused bacterial load reduction in the lungs of mice (29). Overexpression of LipY in M. bovis Bacillus Calmette-Guérin reduced immune protection against Mtb in mice (14). Mutation of Rv2224c, another lipase/esterase, also caused a reduction of bacterial load in mice (10). Recently, we demonstrated that the gene-disrupting mutation of rv1075c, which encodes an esterase, significantly attenuated Mtb intracellular growth in macrophages and reduced bacterial load in the lungs of aerosol infected mice (38). Because these lipases/esterases are important for Mtb infection and growth in hosts, thorough determination of their functions in Mtb lipid/ester catabolism pathways is necessary to expand our knowledge of TB pathogenesis.

Among the 24 lip family lipases/esterases of Mtb, so far only LipE and LipV (34) have been found preferentially hydrolyze medium-chain triglycerols. Of the three lip family proteins (LipD,
LipL, LipP) that were predicted to be evolutionally close to LipE, lipase activities of LipL and LipD have been characterized. LipL prefers a short-chain glyceride butyrate ($K_{cat} = 0.0932$ S$^{-1}$) (15), and LipD preferentially hydrolyzes palmitate ($K_{cat} = 0.02112$ S$^{-1}$) (13). Fatty acids (FAs) released from hydrolysis of medium-chain triglycerols can go through various pathways. For example, FAs released by lipases from medium-chain triglycerols can be synthesized into long-chain FAs by Fas I / II (39). The long-chain FAs are then synthesized into mycolic acids and methyl-branched FAs, which are major components of Mtb cell membrane and cell envelop (40). The medium-chain FAs can be also converted into precursors through \( \beta \)-oxidation pathways to feed into the tricarboxylic acid (TCA) cycle to provide energy for Mtb. We speculate that LipE might be involved in hydrolysis of medium-chain triglycerol to generate critical intermediary metabolites to construct Mtb cell membrane and cell envelop, or to feed into TCA cycle. This hypothesis remains to be further examined.

LipE was identified in the membrane fraction of Mtb using 2D gel electrophoresis combined with liquid chromatography-mass spectrometry (41). In addition, Målen H et al used 2-D gel electrophoresis combined with MALDI-TOF MS and LC coupled MS/MS to profile secreted proteins in Mtb cell filtrates (42), and LipE was not found among the identified total 257 secreted proteins. We also predicted its subcellular location using several web-based software. LipE protein had no predicted signal peptides according to the SignalP 3.0 tool, which indicated that LipE is not secreted via the classical pathway. LipE was predicted to be cell membrane associated or in periplasm by Gpos-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gpos-multi/), Phobius (http://www.ebi.ac.uk/Tools/pfa/phobius/), and PSORTb 3.0.2 (http://www.psort.org/psortb/index.html). Both experimental and in-silico evidences indicate that
LipE is cell membrane-associated, thus can access both external and internal triglycerol to participate lipid metabolism.

Protein-BLAST showed that LipE has homologies to class A β-lactamase-related serine hydrolase with unknown function. It is similar to *Staphylococcus aureus* proteins FmtA and Flp, *Burkholderia gladioli* esterase EstB, and *Bacillus cereus* alkaline D-peptidase. We have also identified that it has a domain (43-398 AA) of β-lactamase using InterProScan5. LipE has three conserved motifs which are observed in penicillin binding proteins and β-lactamases, including three SxxK (97SAK100, 131SHGK134, and 385SSGK388), one SxN (282SSN284), and one HLG box (363HLG365). The SxxK motif identified by MyHits (https://myhits.isb-sib.ch/cgi-bin/motif_scan) is conserved in the carboxylesterase VIII family (43). Our site-directed mutagenesis analysis results demonstrate that the β-lactamase activity and lipase activity of LipE share the same catalytic residues. However, the results of β-lactam antibiotic resistance assay indicate that LipE does not contribute significantly to β-lactam antibiotic resistance of Mtb. As LipE’s amino acid sequence shares homology with DD-peptidases, it could have DD-peptidase activity, which remains to be further explored. Our phylogenetic analysis suggests that LipE is evolutionarily related to LipD and LipL. Like LipE, both LipD and LipL have the typical β-lactamase motif SxxK. However, LipD was found no β-lactamase activity (13). Although LipL was shown to have both lipase and β-lactamase activities, its *Kcat* value for β-lactamase activity was not reported (32). Mtb has another β-lactamase encoding gene *blaC* (*Kcat* = 38 ± 2 S⁻¹ on nitrocefin), which has been shown to possess a TAT signal sequence and can be exported through TAT system. BlaC plays a major role for Mtb resistance to β-lactam antibiotics, as the mutation of *blaC* significantly increase (>16-fold) Mtb’s sensitivity to most of β-lactam antibiotics (44).
We revealed the overall topological organization of LipE through homology modeling. The 3D model of LipE showed that LipE was composed of multiple α helices and β sheets structure, suggesting that LipE belongs to the α/β hydrolase-fold family. We determined that LipE has a 69GHGWG73 motif, which is located in the flexible loop between the second β-sheet and the second α-helix from N-terminus in the predicted 3D structure model. LipE also has a 97SxxK100 motif. Based on the 3D model, we predicted that the active center of LipE as a lipase is composed of Ser$^{97}$, Gly$^{342}$, and His$^{363}$, which is confirmed by the results of the site-directed mutagenesis experiment. These data provide important information for further detailed analysis of the crystal structure of LipE and its lipase/esterase substrates in Mtb lipid/ester metabolism.

In summary, this study demonstrate that Mtb LipE has a lipase/esterase activity and is important for Mtb intracellular survival and in vivo infection. The data from this study imply that LipE is involved in lipid metabolism of Mtb during infection. Further thorough studies to precisely determine the substrates of LipE and its roles in Mtb lipid metabolism and TB pathogenesis will expand our knowledge of how Mtb appropriates host lipids/esters during infection. Identification of critical enzymes in Mtb lipid metabolism will unveil Mtb vulnerabilities to aid drug discovery efforts.
MATERIALS AND METHODS:

Expression vectors, bacterial strains, and media: The Mtb lipE transposon-insertion mutant strain was obtained from BEI Resources. All M. smegmatis and Mtb strains were grown in 7H9 broth (Difco, Detroit, MI) supplemented with 0.5% glycerol, 10% oleic acid dextrose complex without catalase and 0.05% Tween 80 (MOAD-Tw broth), or in Middlebrook 7H9 supplemented with 10% oleic acid dextrose complex without catalase and 15 g/L Bacto agar (MOAD agar, Difco), or on 7H11 selective agar.

Constructing complemented LipE strain: The complementation of the lipE mutant was achieved using a single-copy, site-specific integrating construct that carries the endogenous promoter for lipE. This complementing construct was made by cloning the entire lipE locus along with 78 bp upstream of the translational start and 81 bp downstream of the translational end into the HindIII / BamHI sites under the L5 promoter in the integrating vector pYK13, which expresses a tdTomato under the L5 promoter, and carries a hygromycin resistance gene. Complementation of lipE was confirmed by PCR and DNA sequencing. The wild-type and mutant strains were also transformed with the tdTomato expressing plasmid, pYK13.

Physiochemical properties: Amino acid sequence of LipE of Mtb H37Rv was retrieved from Tuberculist (http://tuberculist.epfl.ch/). Motif determination was carried out using Motif Scan at http://myhits.isb-sib.ch/cgi-bin/motif_scan. Sequence alignment of proteins was carried out by T-COFFEE Espript (http://tcoffee.crg.cat/) with reference to known Lip family lipases of Mtb, and conserved region in a defined box was assigned to the aligned file by Espript3. The evolutionary phylogenetic tree was constructed using Neighbor-Joining algorithm in MEGA7.

Cloning, over-expression, and purification of recombinant LipE: Cloning: DNAs of Rv3775 (LipE) and pMyC vector were extracted using Agarose GelExtract Mini Kit (5-PRIME). The
primers were designed (listed in Table S1) to amplify lipE from genomic DNA of Mtb CDC1551 with a program as follows: denaturation for 5 min at 95°C, then 34 cycles consisting of 30 sec at 95°C, 30 sec at annealing temperature (70°C), and 1 min 30 sec at 72°C, and then 5 min at 72°C for final extension. Further PCR-amplified product was analyzed on a 0.8% agarose gel and purified with DNA clean & concentrator kit (Zymo research). Eluted purified PCR products and DNA of the expression vector pMyC (Addgene) were digested with BamHI and HindIII, respectively. The restriction enzyme-digested pMyC vector fragments were dephosphorylated by phosphatase. After electrophoresis, the DNA bands were cut off the gel and purified, and the gel-purified DNA fragments (vector and amplified lipE PCR product) were mixed and ligated with the T4 DNA ligase. The ligated product was transformed to the chemically competent E. coli DH5α cells to amplify. The cloned lipE was further confirmed by sequencing (Eurofins Genomics). For expression of LipE, the constructed plasmid was isolated and transformed into the expression host M. smegmatis mc² 155 cells. Positive transformants were screened on MOAD agar plates containing 80 μg/mL hygromycin and confirmed by DNA sequencing. Overexpression of LipE: The transformant of M. smegmatis was cultured in MOAD-Tw broth until an optical density at 600 nm (OD<sub>600</sub>) value of 1.5 was reached, induced for 16 h by the addition of 0.2% acetamide, and harvested by centrifugation at 3000 g for 15 min. The pellet was resuspended in cold lysis buffer containing 20 mM Tris-HCl (pH=7.5), 100 mM NaCl, 5% Glycerol, 1 mM imidazole, 10 mM β-mercaptoethanol, and 1X protease inhibitor cocktail (Sigma-Aldrich), and broken with a French Press. The His-tagged rLipE protein was purified by Ni-NTA agarose column-based affinity chromatography and eluted with 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl and concentration-gradient of imidazole. Eluted fractions containing purified rLipE were analyzed by SDS-PAGE gel electrophoresis.
Lipase activity assay: The enzymatic assay of purified recombinant rLipE was performed using different p-nitrophenyl (p-NP) ester substrates such as acetate (p-NP-C2), butyrate (p-NP-C4), hexanoate (p-NP-C6), caprylate (p-NP-C8), caprate (p-NP-C10), laurate (p-NP-C12), myristate (p-NP-C14), palmitate (p-NP-C16), and stearate (p-NP-C18). The 50 mM stock solutions of substrates were prepared in absolute ethanol. The enzymatic reaction mixture was total 350 µL and contained 2 mM p-NP ester substrate, 50 mM phosphate buffer (pH 7.4), 2 mM sodium-deoxycholate, and 10 µg of purified rLipE. The reaction mixture was loaded into a 96-well plate in triplicates with 100 µL / well, and the negative control was the same mixture except that the rLipE was replaced with the buffer. The enzymatic hydrolysis product of all the substrates was p-NP that was quantified by measuring the absorbance at 405 nm kinetically, using a spectrophotometer (Biotek Synergy 2) at various programed temperatures with pathlength correction to 1 cm using a constant = 0.18. During data analysis, the absorbance of the negative control was subtracted from the absorbance of samples with rLipE. One unit of enzyme activity is defined as the amount of enzyme, which generates 1 µmole of p-NP from a p-NP ester substrate per min under the standard assay condition. Enzyme activity of the purified rLipE was determined as a function of substrate concentration (0.01–1.0 mM of p-NP-C6) using standard assay method at 37 °C. The Michaelis–Menten constant (Km), and maximum velocity for the reaction (Vmax) were calculated.

Effects of pH and temperature on enzyme activity: To find out pH optima for rLipE’s esterase activity, the enzyme assay was performed in buffers (50 mM) of different pH values from pH 4.0 to 11.0. Acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0) and Glycine-NaOH buffer (pH 10.0-11.0) were used. To determine optimal temperature of the
enzyme activity, the enzyme assay was performed at various temperatures (25–50°C) for 15 min. Enzyme activity was determined as described above.

**Site-directed mutagenesis:** Site directed mutagenesis was carried out by substituting conserved putative active site residues Ser$^{97}$, Lys$^{100}$, Gly$^{342}$, and His$^{247}$ with alanine using site directed mutagenesis kit, Quickchange II (Agilent, Santa Clara, CA). The primers are listed in Table S1. XL-1-Blue Supercompetent *E. coli* Cells were transformed with plasmids (pMyc backbone) having a confirmed mutant lipE gene. The resulting plasmids were transformed into *M. smegmatis* and the mutant proteins were overexpressed as described above. The mutant rLipEs were purified, checked for enzymatic activity and compared with the wild-type enzyme.

**Thin layer chromatography (TLC):** Trioctanoate (Sigma-Aldrich) at 50 mM was incubated with 100 μL of the wild-type or mutant rLipE (2.5 mg/mL) in 500 μL reaction mix for 30 min. The negative controls were samples without rLipE (only equal volume of buffer). Lipids were extracted with 2:1 of chloroform: methanol followed by gentle mixing and centrifugation at 10,000 rpm for 5 min. The extracted products were run on silica coated TLC plate. The trioctanoate without rLipE and the monoctanoate (Sigma-Aldrich) or dioctanoate (Avanti) served as standards. Chloroform: acetic acid (95: 5) was applied as a mobile phase and primuline (Sigma-Aldrich, 5 mg/100 mL in acetone: water 80:20) stain under 384 nm UV light was used to visualize the products on TLC.

**Resazurin microtiter assay (REMA) for MIC:** Ampicillin, carbenicillin, cefoxitin, ceftriaxone, cephalothin, and rifampicin were purchased from Sigma-Aldrich. The Mtb inoculum was prepared from log-phase Mtb strains (wild-type CDC1551, lipE::Tn mutant, and lipE complemented) growing in MOAD-Tw medium. After adjusting absorbance of bacterial culture to a McFarland tube no. 1, the bacteria were diluted 1:20 with the medium, and 100 μL was used
as an inoculum. After loading with various concentrations of each antibiotic, the plates were covered, sealed in plastic bags, and incubated at 37°C in the normal atmosphere. After 7 days of incubation, 30 μL of resazurin solution (0.02%) was added to each well, incubated overnight at 37°C, and assessed for color development. A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this color change. All MICs were performed in duplicate on at least two independent cultures.

**Prediction of 3D model and refinement of the model:** The templates for homology modeling were selected based on sequence identity and query coverage in SWISS-MODEL workspace. The top three 3D model structures were further screened through PyMol software to select the lowest RMSD value and hydrogen bond distance between the catalytic residues on structural alignment with the template and further energy minimization by YASARA force field.

**LipE gene expression under several stress conditions:** The Mtb CDC1551 strain was grown to mid-log phase and harvested by centrifugation. The bacterial pellet was resuspended in different media for exposure to stressed conditions such as oxidative stress (MOAD-Tw broth with 5 mM H₂O₂), acidic stress (MOAD-Tw broth at pH 4.5), nutrient stress (1X PBS) and temperature stress (40 ºC). These stressed cultures were grown for 15 min or 6 h, respectively, along with a control culture in normal MOAD-Tw broth. After centrifugation, the pellets were resuspended in 1 ml TRIzol to isolate RNA using Direct-zol RNA miniprep kit (Zymo Research) per company’s instruction. The integrity, quantity and quality of RNA samples were validated by an Agilent Bioanalyzer.

Reverse Transcription: RevertAid RT Kit (Thermo Scientific) was used for reverse transcription of RNA to cDNA. The random hexamer primer (1 μL) and template RNA (1 μg) were first
incubated at 65 °C for 5 min, and then chilled on ice. After spinning down, 5X reaction buffer (4 µL), RiboLock RNase Inhibitor (1 µL), 10 mM dNTP mix (2 µL), RevertAid RT (200 U/µL, 1 µL) were added into the reaction in total 20 µL. The reaction mix was incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was then terminated by heating at 70 °C for 5 min. The cDNA samples were diluted to 1:10, 1:20, to 1:100 to further determine the optimal dilution in qPCR.

qPCR: 16S rRNA was an internal control. The reaction was consisted of cDNA 2 µL, primer of lipE, or 16S rRNA, or lipV F and R (listed in the supplemental Table S1) at 10 µM 0.2 µL each, 2X KAPA SYBR FAST qPCR Master mix 5 µL, and water 2.6 µL, total 10 µL. The reaction mix was loaded into a 96-well qPCR plate and amplified by a LightCycler 480 (Roche) at the Molecular Resource Center of UTHSC with a program: denaturation for 5 min at 95°C, then 40 cycles consisting of 10 sec at 95°C, 30 sec at 60°C, and 10 sec at 72°C.

Data Analysis: For each Mtb culture condition, we have three biological replicates. For each cDNA sample, we have 4 technical replicates on the sample 96-well plate. For each condition, ∆Ct equals to that Ct value of lipE (or lipV) subtracts Ct value of 16S rRNA under each condition. ∆∆Ct was calculated as that ∆Ct of each stressed condition subtracts ∆Ct of the normal culture condition. The expression fold change was calculated as $2^{-\Delta\Delta C_{\text{t}}}$.

Cell infection: The tdTomato-expressing Mtb CDC1551 wild-type strain, lipE::Tn mutant strain, and lipE complemented strain were cultured in MOAD-Tw medium until it reached the log-phase and applied to infect macrophages. THP-1 cells or human PBMC derived macrophages were seeded in 96-well transparent bottom culture plates at a concentration of 5x10^4 cells/well. For THP-1, phorbol 12-myristate 13-acetate (PMA) was used to stimulate the cells for three days. The human PBMC acquired from ATCC were seeded into 96-well plates for seven days to allow
monocytes develop into macrophage and to remove the suspending T-cells. The THP-1 or human
PBMC derived macrophages were then infected by bacteria at MOI=10 with 3 replicates for each
strain at 37 °C for 3h. The extracellular bacteria were aspirated, and the infected cells were
washed with 1X PBS, treated with amikacin (200 μg/mL) for additional 2h. After washing with
1X PBS again, the plate was loaded into a microplate reader (Tecan Infinite 200 pro) to measure
fluorescence with tdTomato specific excitation (550 nm) and emission (590 nm) wavelengths. At
day-4 post infection, cell culture medium was removed and replaced with 100 μL 0.1% Triton X-
100 in H2O for 10 min to lyse the cells. Bacteria from lysed cells were titrated and plated on
7H11 agar plates. After 3-4 weeks culture at 37 °C, colonies on the agar plates were counted.
Mouse infection: C3HeB/FeJ female mice (Jackson Laboratory, Bar Harbor, ME) aged 6 to 8
weeks were aerosol infected using the Bioaerosol Nebulizing Generator (BANG) (CH
Technologies Inc., USA) at Regional Biocontainment Laboratory of UTHSC with the Mtb wild-
type, lipE::Tn mutant, and lipE complemented strains from log-phase cultures to deliver a low
dose (5-10 cfu) Mtb in the lungs at day 0. At day 1, two mice from each group were sacrificed to
determine actual delivered bacterial number in the lungs. At day-14, 28 and 56, five mice per
group were sacrificed for tissue collection. Because all the three Mtb strains expressing
tdTomato, we conducted ex vivo fluorescence imaging with the harvested lungs using the IVIS
imaging system. Tissues were then weighed and homogenized to determine CFU using 7H11
selective agar plates.
Ethics Statement: The UTHSC Institutional Animal Care and Use Committee (IACUC)
approved animal care and use protocol 16-102 for all animal experiments in this study. UTHSC
IACUC adheres to the Public Health Service Policy and Animal Welfare Act.
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Potential conflicts of interest

All authors report no potential conflicts.

Abbreviations

Mtb, Mycobacterium tuberculosis; TB, tuberculosis; FAs, fatty acids; p-NP-ester, p-nitrophenyl ester; TLC, thin-layer chromatography; PBMC, peripheral blood mononuclear cell; TAC, tricarboxylic acid cycle.

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REFERENCES:

1. Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, Daffe M, Emile JF, Marchou B, Cardona PJ, de Chastellier C, Altare F. 2008. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence. PLoS Pathog 4:e1000204.

2. Rodriguez JG, Hernandez AC, Helguera-Repetto C, Aguilar Ayala D, Guadarrama-Medina R, Anzola JM, Bustos JR, Zambrano MM, Gonzalez YMJ, Garcia MJ, Del Portillo P. 2014. Global adaptation to a lipid environment triggers the dormancy-related phenotype of Mycobacterium tuberculosis. MBio 5:e01125-14.

3. Ehrt S, Schnappinger D. 2007. Mycobacterium tuberculosis virulence: lipids inside and out. Nat Med 13:284-5.

4. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eglmeier 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, Barrell BG, et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-44.

5. Camus JC, Pryor MJ, Medigue C, Cole ST. 2002. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. Microbiology 148:2967-73.

6. Brust B, Lecoufle M, Tuaillon E, Dedieu L, Canaan S, Valverde V, Kremer L. 2011. Mycobacterium tuberculosis lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis. PLoS One 6:e25078.

7. Canaan S, Maurin D, Chahinian H, Pouilly B, Durousseau C, Frassinetti F, Scappucci-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 271:3953-61.

8. 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 281:3866-75.

9. Jadeja D, Dogra N, Arya S, Singh G, Singh G, Kaur J. 2016. Characterization of LipN (Rv2970c) of Mycobacterium Tuberculosis H37Rv and its Probable Role in Xenobiotic Degradation. J Cell Biochem 117:390-401.

10. Lun S, Bishai WR. 2007. Characterization of a novel cell wall-anchored protein with carboxylesterase activity required for virulence in Mycobacterium tuberculosis. J Biol Chem 282:18348-56.

11. Mishra KC, de Chastellier C, Narayana Y, Bifani P, Brown AK, Besra GS, Katoch VM, Joshi B, Balaji KN, Kremer L. 2008. Functional role of the PE domain and immunogenicity of the Mycobacterium tuberculosis triacylglycerol hydrolase LipY. Infect Immun 76:127-40.

12. Shen G, Singh K, Chandra D, Serveau-Avesque C, Maurin D, Canaan S, Singla R, Behera D, Laal S. 2012. LipC (Rv0220) is an immunogenic cell surface esterase of Mycobacterium tuberculosis. Infect Immun 80:243-53.

13. Singh G, Arya S, xKumar S, Narang D, Kaur J. 2014. Molecular characterization of oxidative stress-inducible LipD of Mycobacterium tuberculosis H37Rv. Curr Microbiol 68:387-96.
14. Singh VK, Srivastava M, Dasgupta A, Singh MP, Srivastava R, Srivastava BS. 2014. Increased virulence of Mycobacterium tuberculosis H37Rv overexpressing LipY in a murine model. Tuberculosis (Edinb) 94:252-61.

15. Singh G, Kumar A, Arya S, Gupta UD, Singh K, Kaur J. 2016. Characterization of a novel esterase Rv1497 of Mycobacterium tuberculosis H37Rv demonstrating beta-lactamase activity. Enzyme Microb Technol 82:180-90.

16. 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.

17. Kumar A, Sharma A, Kaur G, Makkar P, Kaur J. 2016. Functional characterization of hypothetical proteins of Mycobacterium tuberculosis with possible esterase/lipase signature: a cumulative in silico and in vitro approach. J Biomol Struct Dyn doi:10.1080/07391102.2016.1174738:1-18.

18. Glickman MS, Cox JS, Jacobs WR. 2000. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of Mycobacterium tuberculosis.

19. Murphy HN, Stewart GR, Mischenko VV, Apt AS, Harris R, McAlister MS, Driscoll PC, Young DB, Robertson BD. 2005. The OtsAB pathway is essential for trehalose biosynthesis in Mycobacterium tuberculosis. J Biol Chem 280:14524-9.

20. Reed MB, Domenech P, Manca C, Su H, Barczak AK, Kreiswirth BN, Kaplan G, Barry CE, 3rd. 2004. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. Nature 431:84-7.
21. Rindi L, Fattorini L, Bonanni D, Iona E, Freer G, Tan D, Deho G, Orefici G, Garzelli C. 2002. Involvement of the fadD33 gene in the growth of Mycobacterium tuberculosis in the liver of BALB/c mice. Microbiology 148:3873-80.

22. McKinney JD, zu Bentrup KH, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR, Russell DG. 2000. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406:735-738.

23. Raynaud C, Guilhot C, Rauzier J, Bordat Y, Pellicc V, Manganelli R, Smith I, Gicquel B, Jackson M. 2002. Phospholipases C are involved in the virulence of Mycobacterium tuberculosis. Mol Microbiol 45:203-217.

24. Chang JC, Miner MD, Pandey AK, Gill WP, Harik NS, Sassetti CM, Sherman DR. 2009. igr Genes and Mycobacterium tuberculosis cholesterol metabolism. J Bacteriol 191:5232-9.

25. Gioffre A, Infante E, Aguilar D, Santangelo MP, Klepp L, Amadio A, Meikle V, Etchechoury I, Romano MI, Cataldi A, Hernandez RP, Bigi F. 2005. Mutation in mce operons attenuates Mycobacterium tuberculosis virulence. Microbes Infect 7:325-34.

26. Marjanovic O, Miyata T, Goodridge A, Kendall LV, Riley LW. 2010. Mce2 operon mutant strain of Mycobacterium tuberculosis is attenuated in C57BL/6 mice. Tuberculosis (Edinb) 90:50-6.

27. Senaratne RH, Sidders B, Sequeira P, Saunders G, Dunphy K, Marjanovic O, Reader JR, Lima P, Chan S, Kendall S, McFadden J, Riley LW. 2008. Mycobacterium tuberculosis strains disrupted in mce3 and mce4 operons are attenuated in mice. J Med Microbiol 57:164-70.
28. Nazarova EV, Montague CR, La T, Wilburn KM, Sukumar N, Lee W, Caldwell S, Russell DG, VanderVen BC. 2017. Rv3723/LucA coordinates fatty acid and cholesterol uptake in Mycobacterium tuberculosis. Elife 6.

29. Camacho LR, 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 34:257-67.

30. Saviola B, Woolwine SC, Bishai WR. 2003. Isolation of acid-inducible genes of Mycobacterium tuberculosis with the use of recombinase-based in vivo expression technology. Infect Immun 71:1379-88.

31. Dutta NK, Mehra S, Didier PJ, Roy CJ, Doyle LA, Alvarez X, Ratterree M, Be NA, Lamichhane G, Jain SK, Lacey MR, Lackner AA, Kaushal D. 2010. Genetic requirements for the survival of tubercle bacilli in primates. J Infect Dis 201:1743-52.

32. Cao J, Dang G, Li H, Li T, Yue Z, Li N, Liu Y, Liu S, Chen L. 2015. Identification and Characterization of Lipase Activity and Immunogenicity of LipL from Mycobacterium tuberculosis. PLoS One 10:e0138151.

33. Flannagan RS, Cosio G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7:355-66.

34. Singh G, Arya S, Narang D, Jadeja D, Singh G, Gupta UD, Singh K, Kaur J. 2014. Characterization of an acid inducible lipase Rv3203 from Mycobacterium tuberculosis H37Rv. Mol Biol Rep 41:285-96.

35. Lamichhane G, Zignol M, Blades NJ, Geiman DE, Dougherty A, Grosset J, Broman KW, Bishai WR. 2003. A postgenomic method for predicting essential genes at subsaturation.
levels of mutagenesis: application to Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 100:7213-7218.

Kong Y, Yang D, Cirillo SL, Li S, Akin A, Francis KP, Maloney T, Cirillo JD. 2016. Application of fluorescent protein expressing strains to evaluation of anti-tuberculosis therapeutic efficacy in vitro and in vivo. PLoS One 11:e0149972.

Pichugin AV, Yan BS, Sloutsky A, Kobzik L, Kramnik I. 2009. Dominant role of the sst1 locus in pathogenesis of necrotizing lung granulomas during chronic tuberculosis infection and reactivation in genetically resistant hosts. Am J Pathol 174:2190-2101.

Yang D, He X, Li S, Liu J, Stabenow J, Zalduondo L, White S, Kong Y. 2019. Rv1075c of Mycobacterium tuberculosis is a GDSL-Like Esterase and is Important for Intracellular Survival. J Infect Dis doi:10.1093/infdis/jiz169.

Smith JL, Sherman DH. 2008. An enzyme assembly line. Science 321:1304-5.

Kremer L, de Chastellier C, Dobson G, Gibson KJ, Bifani P, Balor S, Gorvel JP, Locht C, Minnikin DE, Besra GS. 2005. Identification and structural characterization of an unusual mycobacterial monomeromycolyl-diacylglycerol. Mol Microbiol 57:1113-26.

Gu S, Chen J, Dobos KM, Bradbury EM, Belisle JT, Chen X. 2003. Comprehensive proteomic profiling of the membrane constituents of a Mycobacterium tuberculosis strain. Mol Cell Proteomics 2:1284-96.

Malen H, Berven FS, Fladmark KE, Wiker HG. 2007. Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. Proteomics 7:1702-18.

Arpigny JL, Jaeger KE. 1999. Bacterial lipolytic enzymes: classification and properties.

Biochem J 343 Pt 1:177-83.
Flores AR, Parsons LM, Pavelka MS, Jr. 2005. Genetic analysis of the beta-lactamases of Mycobacterium tuberculosis and Mycobacterium smegmatis and susceptibility to beta-lactam antibiotics. Microbiology 151:521-32.
Fig. 1. Protein sequence analysis of LipE and predicted 3D structure model of LipE. (A) Protein sequence alignment of LipE with LipD, LipL and LipP, and Esterase A (PDB:5gkv.1) from Caulobacter crescentus. The putative active triad amino acids of LipE was indicated by blue triangles. (B) Predicted LipE 3D structure model. (C) Zoom in the putative active triad area in the 3D structure model. Distances between atoms on the putative active triad amino acids are shown in yellow dashes (unit=Å). (D) Alignment of LipE structure with the structure of Caulobacter crescentus Esterase A (PDB:5gkv.1). Cyan: LipE; Pink: 5GKV.1. (E) LipE 3D structure surface view. Red color represents the predicted active triad.

Fig. 2. Purification of rLipE and analysis of LipE lipase/esterase activity. (A) SDS-PAGE analysis of rLipE eluted by various concentrations of imidazole from His-tag Ni-column. Lane 1: protein molecular weight marker (PageRulerM); 2: protein extracts run through the Ni-column with 40 mM imidazole buffer; 3-6: samples eluted from Ni-column with 125, 250, and 500 mM imidazole, respectively. (B) Lipase relative activity of rLipE towards p-NP esters with various chain lengths (C6: caproate; C8, caprylate; C10: caprate; C12: laurate; and C14: myristate). Activity with p-NP-C6 was set as 100%. (C) The effect of temperature on lipase activity of rLipE using p-NP-C6 as a substrate at pH=7.4. Activity at 40 °C was set as 100%. (D) The effect of pH on lipase activity of rLipE using p-NP-C6 as a substrate at 37 °C. Activity at pH=7.4 was set as 100%. (E) Specific activity (U / mg) of rLipE over a series of p-NP-C6 concentrations. (F) Lineweaver-Burk (double-reciprocal) plot of specific activity\(^{-1}\) against p-NP-C6 concentration\(^{-1}\). The values represent the means ± SD of three independent experiments. (G) Verification of the active triad by site-directed mutagenesis. The wild-type or site-directed mutant rLipE was incubated with p-NP-C6 at 37 °C and pH=7.4. Activity of the wild-type rLipE was set as 100%.
**** P<0.0001 (One-way ANOVA, followed by Dunnett’s multiple comparison test). The values represent the means ± SD of three independent experiments.

Fig. 3. **rLipE is capable of hydrolyzing trioctanoate.** TLC analysis of hydrolysis of trioctanoate by rLipE. Lane 1: Trioctanoate + wild-type rLipE; 2: Trioctanoate + S97A rLipE; 3: Trioctanoate + G342A rLipE; 4: Trioctanoate + H363A rLipE; 5: Trioctanoate standard; 6: Dioctanoate standard; and 7: Monoctanoate standard. Incubation time: 30 min.

Fig. 4. **rLipE hydrolyzes fluorocillin green, a β-lactamase substrate.** (A) Kinetic of rLipE on fluorocillin green. Five concentrations (2.5-40 μg/mL) of fluorocillin green were incubated with rLipE (10 μg) at 37 °C. Kinetic of rLipE on fluorocillin green was measured at excitation wavelength 485 nm and emission wavelength 528 nm. (B) Determination of LipE active residuals for its β-lactamase activity by site-directed mutagenesis. Wild-type or mutant rLipE (10 μg) was incubated with 10 μg/mL fluorocillin green at 37 °C for 2 hours. **** P<0.0001 (One-way ANOVA, followed by Dunnett’s multiple comparison test). The values represent the means ± SD of three independent experiments.

Fig. 5. **Gene expression of lipE under stressed conditions.** Mtb RNA was extracted at 15 min or 6-h after inoculation under the tested conditions. 16S-rRNA was used as an internal control to normalize transcript level of lipE under each stressed and normal condition. The gene expression ratio was calculated as: the normalized transcript level under the stressed condition / the normalized transcript level under the normal culture condition (MOAD-Tw medium at 37 °C). The conditions that induced lipE expression significantly were marked with **** (P<0.0001). LipV served as a control, because it was reported to be only upregulated under the acidic stress condition. Statistical significance (P values) for comparisons between groups are shown in Table S2.
Fig. 6. The gene-disrupting mutation of lipE leads to attenuated growth in the THP-1 cell line (A & C) and human PBMC derived macrophage (B & D). WT: wild-type; Comp.: complemented. MOI=10. Intracellular growth ratio = FI at each time point post infection / FI at 0-h after removing extracellular bacteria. Intracellular growth ratios of strains were measured daily for four days (A & B), and CFU enumeration was done at 96-h after removing extracellular bacteria (C & D). The values represent the means ± SD of three experiments. **** P<0.0001; *** P<0.001; ** P<0.01; and * P<0.05 (One-way ANOVA, followed by Dunnett’s multiple comparison test).

Fig. 7. The gene-disrupting mutation of lipE leads to a bacterial load reduction in Mtb infected mice. (A) CFU of the lungs of mice infected by the wild-type, lipE::Tn mutant and complemented Mtb strains at day-1, -14, -28 and -56. (B) Ex vivo imaging of the lungs of mice infected by the wild type, lipE::Tn, and complemented Mtb strains at day-28 and -56 post infection. (C) Quantitative analysis ex vivo imaging data. Five mice in each group were sacrificed at each time point. WT: wild-type; Comp.: complemented. **** P<0.0001; ** P<0.01; and * P<0.05 (One-way ANOVA, followed by Dunnett’s multiple comparison test).
Table 1. MICs (µg/mL) of β-lactam antibiotics and rifampicin (RIF) against the wild-type, *lipE::Tn* mutant, and *lipE* complemented Mtb strains.

| Drugs          | Wild-type | *lipE::Tn* mutant | Complemented |
|----------------|-----------|------------------|--------------|
| Ampicillin     | 240       | 120              | 240          |
| Carbenicillin  | 240       | 240              | 240          |
| Cefoxitin      | 15        | 7.5              | 15           |
| Ceftriaxone    | 30        | 7.5              | 30           |
| Cephalothin    | 30        | 7.5              | 30           |
| RIF            | 0.25      | 0.25             | 0.25         |
Fig. 1.

| LipE | RPDFATG...EMLFRGQ...MGSAK...YCPFGPNAPAAGRHGVINVAKNADERAADSSG |
| LipD | WDLMNLG...LPFTGKQ...QSSFPV...LLEGCHGGG...GWDADECGSAPFG |
| LipL | LGLNLNL...PLNHR...BGMF...QCN...VMNPQHCNGG...GWQDERDGAQA |
| LipP | DDLYSGPR...ADHGMG...MLNQRG...VQGP...NPISGRGGCGGG...GFVQCEHRQGYA |
| EstA | RDVLVR...MVIELYAGQ...RMNNEPNFYVGPG...TAEAEOBQGGAGCAFADEIRGVSGA |

**A**

![Sequence alignment with protein structures](image_A.png)

**B**

![3D structure of LipE and LipD](image_B.png)

**C**

![3D structure of LipL and LipP](image_C.png)

**D**

![3D structure of EstA](image_D.png)

**E**

![3D structure with protein interaction](image_E.png)
Fig. 2.

A. A gel image with bands labeled rLipE.

B. A bar graph showing relative activity (%) for different alcohols (C6 to C14).

C. A line graph showing relative activity (%) vs. temperature (°C).

D. A line graph showing relative activity (%) vs. pH.

E. A line graph showing specific activity vs. concentration (mM).

F. A line graph showing specific activity (mM)^{-1} vs. concentration (mM)^{-1}.

G. A bar graph showing relative activity (%) for different mutants.

R² = 0.98
Fig. 3.
Fig. 4.

**A**

![Graph showing kinetic 5 conc.](image)

**B**

![Bar chart showing relative activity (%)](image)
Fig. 5.

\[ H_2O_2 \text{ pH = 4.5 PBS } 40^\circ C \]

\[ \text{Ratio (stress/normal)} \]

\[ \text{H}_2\text{O}_2 \quad \text{pH = 4.5} \quad \text{PBS} \quad 40^\circ C \]

\[ \text{lip E} \]

\[ \text{15 min} \quad \text{6-h} \]

\[ ***** \quad **** \]

\[ \text{lip V} \]

\[ \text{15 min} \quad \text{6-h} \]

\[ **** \]
Fig. 6.
Fig. 7.

**A**

Bar graph showing the comparison of WT, lipE::Tn, and Comp. for days 1, 14, 28, and 56.

**B**

Images showing fluorescence of D28 and D56 for WT, lipE::Tn, and Comp. with color scale indicating p/sec/cm²/sr/μW/cm².

**C**

Fluorescence comparison for D28 and D56 showing significant differences between WT, lipE::Tn, and Comp. with asterisks indicating levels of significance.