Mycobacterium tuberculosis Rv3802c Encodes a Phospholipase/Thioesterase and Is Inhibited by the Antimycobacterial Agent Tetrahydrolipstatin

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Abstract

The cell wall of M. tuberculosis is central to its success as a pathogen. Mycolic acids are key components of this cell wall. The genes involved in joining the α and mero mycolates are located in a cluster, beginning with Rv3799c and extending at least until Rv3804c. The role of each enzyme encoded by these five genes is fairly well understood, except for Rv3802c. Rv3802 is one of seven putative cutinases encoded by the genome of M. tuberculosis. In phytopathogens, cutinases hydrolyze the waxy layer of plants, cutin. In a strictly mammalian pathogen, such as M. tuberculosis, it is likely that these proteins perform a different function. Of the seven, we chose to focus on Rv3802c because of its location in a mycolic acid synthesis gene cluster, its putative essentiality, its ubiquitous presence in actinomycetes, and its conservation in the minimal genome of Mycobacterium leprae. We expressed Rv3802 in Escherichia coli and purified the enzymatically active form. We probed its activities and inhibitors characterizing those relevant to its possible role in mycolic acid biosynthesis. In addition to its reported phospholipase A activity, Rv3802 has significant thioesterase activity, and it is inhibited by tetrahydrolipstatin (THL). THL is a described anti-tuberculous compound with an unknown mechanism, but it reportedly targets cell wall synthesis. Taken together, these data circumstantially support a role for Rv3802 in mycolic acid synthesis and, as the cell wall is integral to M. tuberculosis pathogenesis, identification of a novel cell wall enzyme and its inhibition has therapeutic and diagnostic implications.

Introduction

M. tuberculosis infects one third of the world, makes over 8 million ill each year, and kills 1.8 million, 450,000 of whom are children [1]. Though one of the oldest known human pathogens, our ability to combat spread of this disease remains insufficient [2]. Key to the success of the tuberculosis bacillus is its uniquely complex lipid rich cell wall, and cell wall synthesis pathways are current targets for drug development [3–6]. The cell wall of mycobacteria is considered a bilayer, and the lipids integral to the bilayer are the mycolic acids. Just exterior to the cell membrane lies the mycolyl-arabinogalactan-peptidoglycan complex (mAGP). This complex forms the stable scaffolding for the outer component of surface-exposed lipids and glycolipids, such as trehalose monomycoside (TMM) and dimycolate (TDM). The mycolic acids of these glycolipids are noncovalently intercalated with the mAGP.

Abstract

The role of each enzyme encoded by these five genes is fairly well understood, except for Rv3802c, though these five genes do not account for all the necessary steps in the pathway [7]. A summary is presented in Figure 1. In brief, FadD32 (fatty acid dehydrogenase, Rv3901c) and AccD4 (Rv3799c), along with the other acyl-CoA carboxylases, AccA3 and AccD5, participate in deriving meroacyl-AMP (from FAS-II) and 2-carboxyl-C26-S-CoA (from FAS-I), respectively. These are attached via thioester bonds to phosphopentathienes on the acyl carrier domains of Pks13 (polyketide synthase, Rv3800c), a multidomain protein which, via Claissen condensation, creates a mature mycolic acid. A reduction step is needed and one enzyme so far, CmrA, has been identified for this role [8]. The deposition and export of the mycolates is less well understood. A mature mycolic acid is eventually transferred either to arabinogalactan or to trehalose or arabinogalactan are located in a gene cluster from Rv3799c to at least Rv3804c (Figure 1). The role of each enzyme encoded by these five genes is fairly well understood, except for the product of Rv3802c, though these five genes do not account for all the necessary steps in the pathway [7]. A summary is presented in Figure 2. In brief, FadD32 (fatty acid dehydrogenase, Rv3901c) and AccD4 (Rv3799c), along with the other acyl-CoA carboxylases, AccA3 and AccD5, participate in deriving meroacyl-AMP (from FAS-II) and 2-carboxyl-C26-S-CoA (from FAS-I), respectively. These are attached via thioester bonds to phosphopentathienes on the acyl carrier domains of Pks13 (polyketide synthase, Rv3800c), a multidomain protein which, via Claissen condensation, creates a mature mycolic acid. A reduction step is needed and one enzyme so far, CmrA, has been identified for this role [8]. The deposition and export of the mycolates is less well understood. A mature mycolic acid is eventually transferred either to arabinogalactan (to form mAGP) in part by the Ag85 proteins, or to an α,α-trehalose-6-phosphate (T6P, leading to TMM) by an unknown mycolyltransferase [9]. A phospholipid carrier, 6-0-mycobyl-a-D-mannopyranosyl-1-monophosphoheptaprenol (Mye-PL), is reported to be involved in Mycobacterium smegmatis[10]. For TDM formation, a second mycolic acid is added to TMM by the antigen 85 complex
mycolyltransferases, including Ag85A (Rv3804c) [11]. In this study we investigated the possible contributions of the Rv3802c gene product to the mycolic acid synthesis pathway. Our data demonstrate that the products encoded by Rv3802c and the non-orthologous but homologue MSMEG_1403 possess both phospholipase A (PLA) activity and thioesterase activity. These activities are consistent with a role in mycolic acid biosynthesis as this pathway involves multiple ester and thioester bonds. In addition, the Rv3802c gene product, but not that of the distant homologue MSMEG_1403 in M. smegmatis, is inhibited by the known human fatty acid synthase thioesterase (FASTE) domain inhibitor, THL. THL has been demonstrated to decrease mycolic acid synthesis, leading to defects in the mycobacterial cell wall, though the targets of its antimycobacterial action were unknown. Though these lines of evidence are circumstantial, taken together with the genetic evidence, it has been found in the membrane fraction [20,21].

Expression and purification of recombinant proteins M. tuberculosis Rv3802, its mutants, and M. smegmatis mc^2155 MSMEG_1403 in E. coli

The genes Rv3802c and MSMEG_1403 were PCR-amplified from their respective genomes without their secretion signals and placed under the control of the IPTG inducible T7 promoter in the expression vector pET23a. The vector derived 6×His tag was not used in lieu of an engineered streptavidin tag, and expression in each recombinant strain was verified using an anti-streptavidin antibody (Figure 4). The product of MSMEG_1403 is not the ortholog of Rv3802, rather it is a homologous protein we purified from M. smegmatis culture supernatant for its PLA activity [22]. Though not the focus of this manuscript, we include information on MSMEG_1403 here because it confirms that cutinases have PLA and TE activity, novel activities for this class of enzymes. In addition, it demonstrates that a non-orthologous cutinase, MSMEG_1403, is not inhibited by THL, which is significant because growth of M. smegmatis in culture also is not inhibited by THL [23]. Amino acid changes in Rv3802 were made in serines 86, 87 and 175. Each was changed to a glutamic acid. Ser175 is predicted to be the catalytic serine. Either Ser86 or Ser87 is predicted to stabilize the oxygen hole, based on publications with F. solani cutinase Ser42; these were mutated with the goal of finding a partially active mutant [24]. Solubility was increased with use of sarcosyl for membrane disruption. Purity was assessed to be at least 95% on an SDS-polyacrylamide gel stained with a technique sensitive to 10 ng of protein (Figure 4). Notably, MSMEG_1403 electrophoreses larger on the gel than Rv3802, though Rv3802 is predicted to be 32.5 kD and MSMEG_1403 30 kD. Reasons for this are unclear, but it is a consistent finding. In addition, with MSMEG_1403, dimers and trimers as well as monomers are often visible.
Activity of purified proteins *M. tuberculosis* Rv3802 and *M. smegmatis* mc²155 MSMEG_1403 on various substrates

In order to understand its location in the cell wall pathway, various substrates and activities were explored. This included assays for esterase, lipase, phospholipase, thioesterase and mycolyltransferase activities.

Phospholipids are integral to both host membranes and the mycobacterial cell wall, and it is known that exogenous radiolabeled 14C-phospholipids are incorporated into the cell wall [25,26]. In theory, this is through fatty acid incorporation into the FAS-II pathway, though complete catabolism may also occur. In any event, PLA activity is a necessary part of this process, thus we further investigated the PLA activity of purified Rv3802 and MSMEG_1403. Of note, we described PLA activity previously, but on partially purified native protein rather than purified exogenous protein [22]. To investigate the ability of Rv3802 and MSMEG_1403 to hydrolyze host and mycobacterial phospholipids, hydrolysis of 14C-radiolabeled phospholipids was assessed by TLC. Rv3802 and MSMEG_1403 were active in a PLA-type fashion (hydrolysis to respective lysophospholipid and fatty acid) on phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, but not sphingomyelin (Figure 5). The enzymes were able to hydrolyze both palmitic acid and arachidonic acid containing phospholipids.
Figure 3. Clustal W alignment of Rv3802 with its orthologs in mycobacteria demonstrating high conservation. Cutinase motif is boxed with asterisk over putative catalytic site amino acids. doi:10.1371/journal.pone.0004281.g003

Figure 4. Products of Rv3802c and MSMEG_1403 expression in E. coli. Left: polyacrylamide gel visualized with Sypro Orange™. Right: immunoblot with streptavidin antibody. doi:10.1371/journal.pone.0004281.g004
Because these proteins are annotated as cutinases, hydrolysis of nitrophenyl butyrate (NPB) was investigated as a surrogate for cutinase activity. This is essentially an esterase activity with colorimetric detection of the nitrophenyl group freed from NPB. Both enzymes were active esterases, though the turnover rates were relatively slow (Table 1).

Though Rv3802 may function for hydrolysis only, evaluation of the mycolic acid synthesis pathway suggests a possible role as a transferase (Figure 2). Rv3802 and Antigen85A (Rv3804), a known mycolyltranferase, are both α/β-hydrolase fold enzymes. The acyl binding pocket of Ag85A functions to transfer the second mycolic acid onto TMM to from TDM. We therefore investigated acyltransferase activity of Rv3802 and MSMEG_1403. Various acyl donors were paired with acceptors in enzyme assays. First, to investigate transacylation of trehalose (similar to Ag85) 14C-trehalose was used as the acceptor, and cell wall lipids or crude cell wall extract (with lipid and protein) were used for donation; no transacylation was detected except with the positive control, Ag85C (data not shown). T6P is not currently commercially available, thus a similar assay was done with the acceptors mannose-6-phosphate and glycerol-6-phosphate, with no observed acylated product. These same acceptors were used but the donor changed to 14C-P-CoA (thioester bond) or 14C-DPPC (ester bond), and again no acylation occurred, though hydrolysis of the donors did occur. 14C-P-CoA and 14C-DPPC were again used as donors, with crude cell wall extract (lipids and proteins, ATP also added) used for the acceptor; no new acylated products emerged. 14C-P-CoA and 14C-DPPC were again used as donors, with crude cell wall extract (lipids and proteins, ATP also added) used for the acceptor; no new transacylation was observed. Transacylation assays were repeated several times with different amounts of donor, acceptor and enzyme, and different amounts of time from 1 to 72 H. The only result for Rv3802 and MSMEG_1403 in all assays was the release of fatty acid (not shown).

As a more global cell wall assay, M. smegmatis mc2 155 cell wall was radiolabeled with 14C-acetate, and the outer cell wall was extracted as in methods. This labeled cell wall was assayed with Rv3802 and MSMEG_1403 protein, and diminution of a labeled lipid as well as appearance of new lipid with a similar retention

**Table 1.** Kinetics of Rv3802 and Rv3802 S87DE mutant.

| Enzyme and Substrate assayed | Km, mM | Vmax Moles[S] min⁻¹ mg⁻¹ | Kcat sec⁻¹ | Specificity constant M⁻¹ sec⁻¹ |
|-----------------------------|-------|-------------------------|------------|-------------------------------|
| Rv3802                      | 23.52+/−3.78 | 1.62+/−0.08×10⁻⁸ | 8.81+/−0.31×10⁻³ | 0.37+/−0.01 |
| NPB                         | 12.91+/−2.53 | 8.76+/−0.55×10⁻⁹ | 4.77+/−0.28×10⁻³ | 0.37+/−0.02 |
| S87DE                       |        |                        |            |                               |
| NPB                         | 0.017+/−0.003 | 1.35+/−0.04×10⁻⁷ | 7.33+/−0.62×10⁻² | 37.00+/−11.08 |
| Palmitoyl-S-CoA             | 2.28+/−1.06 | 1.11+/−0.25×10⁻⁷ | 8.45+/−2.53×10⁻² | 37.00+/−11.08 |
| Decanoyl-S-CoA              |        |                        |            |                               |

Rv3802 S86DE and S175DE are inactive in both assays, as is snake venom PLA2. NPB substrate represents an ester bond, while acyl-CoA substrates represent thioester bonds.

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factor (Rf) to known 14C-palmitic acid resulted. Mass spectrometry of the apparent substrate revealed it as phosphatidylinositol mannoside 2 (PIM2); this was purified by normal phase chromatography on a silica column, and hydrolysis by Rv3802 and MSMEG_1403 confirmed (Figure 6). Based on Rf in this solvent, the PIM hydrolysis product is consistent with a fatty acid, including tuberculostearic acid[27]. This result was not surprising given that we have demonstrated that these enzymes possess PLA activity.

We investigated the thioesterase activity of these enzymes because of the possibility that Rv3802 and its orthologs hydrolyze the thioester bond of nascent mycolic acids on Pks13 as the terminal event in synthesis or, alternatively, that the Rv3802c gene product could be a “proof reading” thioesterase. This assay uses acyl-CoA as the thioester substrate, with colorimetric detection of TNB2 formed when DTNB reacts with the hydrolyzed product HS-CoA[28]. Both M. tuberculosis Rv3802 and M. smegmatis MSMEG_1403 protein were active as thioesterases (Table 1).

For the kinetic analysis of Rv3802, the time-dependent hydrolysis of palmitoyl CoA (P-CoA) and decanoyl CoA (D-CoA) was measured for thioesterase activity, and of NPB for esterase activity (see Table 1). These kinetic values are similar to those obtained with other thioesterases. While Rv3802 appears to favor thioester bonds over ester bonds considering the kinetic values, the difference in acyl chain length may account for this, as Rv3802 has a higher affinity for hexadecanoyl than decanoyl thioesters. A comparison between similarly acylated thioesters and esters would be of interest, but we are limited by available substrates and solubility of compounds, as the nitrophenyl esters are less soluble in aqueous solvents than CoA thioesters, and thus one would end up comparing a soluble substrate to a micellar substrate, which may influence results [29]. Rv3802 was not active on acetyl-CoA. The Rv3802 mutants of S175ΔE and S86ΔE were essentially inactive, while the S87ΔE mutant is partially active in both assays (Table 1). Snake venom PLA2 was not active in either the colorimetric esterase or thioesterase assays (data not shown).

Inhibition of M. tuberculosis Rv3802 and non-inhibition of M. smegmatis mc2155 MSMEG_1403 with THL

THL is reported to inhibit the thioesterase domain of human FAS, thus we investigated the ability of THL to inhibit M. tuberculosis Rv3802. Inhibition of PLA activity is demonstrated in Figure 7. The kinetics of inhibition were performed using the thioesterase assay. Complete inhibition was seen at THL concentration of 3.125 μM, a molar excess of 6.7:1 inhibitor to enzyme. The apparent Ki and IC50 for THL against Rv3802 are 5 and 250 nm, respectively, which are lower than against human FASTE, for which the apparent Ki and IC50 are reported at 100 nm and 1.35±0.34 μM, respectively [30]. It is significantly better than the 200:1 molar excess needed for partial inhibition reported against the only reported mycobacterial target of THL, Rv0183[30–32]. Interestingly, THL did not inhibit MSMEG_1403 up to THL concentrations of 25 μM, a 500 M excess.

Discussion

In this report we described the heterologous expression, purification and enzymatic characterization of an annotated cutinase, Rv3802, from a mammalian pathogen. Though the proteins encoded by M. tuberculosis cutinases, including Rv3802, have been immunologically characterized, their function in organisms that do not encounter cutin has not been elucidated [33]. We previously attributed mycobacterial PLA activity to the
cutinases, and in this manuscript additionally ascribe thioesterase activity. Logical potential uses for these activities by a mammalian pathogen include host lipid hydrolysis for carbon scavenging, or mycobacterial cell wall construction/remodeling. As there are seven annotated cutinases in *M. tuberculosis*, it may be they serve a variety of functions. The function of *Rv3802c* and its orthologs is suggested by its location in a gene cluster for mycolic acid synthesis. In addition, the importance of *Rv3802c* is supported by its putative essentiality, its ubiquitous presence in actinomycetes, and its unique conservation in the minimal genome of *M. lprae*. To date, all of the genes surrounding *Rv3802c* are demonstrated to serve vital functions in mycolic acid synthesis. This genetic data implicating a role in mycolic acid synthesis is further supported by *Rv3802c*: (i) membership in the α/β-hydrolase fold family with known PLAs, mycolyltransferases and thioesterases; (ii) possession of thioesterase activity; and (iii) enzymatic inhibition by a known mycolic acid synthesis inhibitor, THL.

The compound THL is sold over the counter as the weight loss agent Alli^®^M. It inhibits pancreatic lipase so that long chain fatty acids cannot be absorbed. During activity based screening, THL was noted to be a selective inhibitor of the TE domain on human FAS [30]. FAS is a modular protein like Pks13. FAS has little effect on normal cells because they take up dietary lipids but, in tumor cells, FAS is upregulated and contributes to angiogenesis; thus THL is currently a lead compound in the development of anticancer drugs [28,34]. This development is focusing on creating more absorbable and specific forms [30,35]. THL has been demonstrated to inhibit *M. tuberculosis* growth at <30 μg/mL, decreasing the production of mycolic acids with concomitant defects in the cell wall, though the target(s) were undefined [23]. Possible targets of THL, including the diacylglycerol acyltransferase *Rv3130* and the FAS-I and II pathways were investigated, but none were identified as a target [23]. A monoglyceride lipase, *Rv0183*, was later identified as a possible target, but a 200:1 molar excess of inhibitor to enzyme was needed to see an effect [32]. Based on our knowledge that *Rv3802* is an α/β-hydrolase fold with TE activity, and the structure of THL, we investigated the ability of THL to inhibit recombinant *Rv3802* and found it to inhibit at nanomolar concentrations, as described. The mechanism of inhibition is assumed to be similar to the mechanism of human FASTE inhibition. For human FASTE, THL is demonstrated to inhibit competitively, with the palmitic core of THL fitting into the hydrophobic substrate binding pocket and the hexanoyl tail packing against the catalytic histidine, significantly delaying hydrolysis (Figure 7) [31,34]. Eventually it is hydrolyzed, thus inhibition is reversible. The inhibition of an enzyme whose gene is located in the mycolic acid synthesis gene cluster, together with previously published observations that THL affects the cell wall and decreases mycolic acids, supports a cause and effect relationship between THL and *Rv3802*. Although we demonstrated that THL inhibits *Rv3802* in assays, this does not confirm that *Rv3802* is the primary target of THL in culture. It is likely that THL inhibits other enzymes. In any case, the knowledge that THL inhibits *Rv3802* at least gives a framework for exploring inhibition of *Rv3802* and other THL targets further in *M. tuberculosis*.

Though further investigation will be required to pinpoint the exact function of *Rv3802*, examination of the mycolic acid synthetic pathway suggests a role as a Pks associated thioesterase or as a mycolyltransferase. Though we could not demonstrate mycolyltransferase activity, our data is limited by the need to use surrogate substrates and acceptors, thus this remains a distinct possibility. There are multiple unknown mycolyltransferases required in the pathway, and similarities between the known mycolyltransferase Ag85 proteins and *Rv3802* are hard to ignore. However, the unusual ability to hydrolyze thioesters strongly suggests some type of interaction with nascent mycolic acids while docked via thioester bonds on Pks13.

Interaction with Pks13 could be in the form of an exogenous type 1 thioesterase, (TE1) or as an exogenous type 2 thioesterase (TE2) [37]. Type I polyketide synthases (PKSs) are modular, "assembly line"-like enzymes responsible for the steps in the biosynthesis of polyketides and mycolic acids [38]. Pks13 in *M. tuberculosis* has all the necessary modules to produce a mature mycolic acid: one acyltransferase domain for loading and transfer of selected acyl chains to the phosphopentenine (PP) binding motif of the acyl-S-carrier protein (ACP) domain; two ACPs for thioester binding, via a PP, of the mecorac chain from FadD32 and the 2 carboxyl-C26 chain from AccD4, AccA3 and AccD5; a ketoacetyl synthase domain for Claisen-type condensation; and one TE1 domain for chain termination and hydrolysis. The reduction event to form the mature mycolic acid may be catalyzed by more than one protein, but to date CmA has been identified [8]. The mature mycolic acid can then be exported and transferred to the mycolate containing components of the mycobacterial cell wall, via unknown mycolyltransferases. However, it has been proposed that the TE1 on the C terminal domain of Pks13 does not liberate the mature mycolic acid because the presence of free mycolic acid would be unwieldy for the cell [7]. A solution to this problem is to have an external TE1 with mycolyltransferase activity. In theory, *Rv3802* could fill this role, though admittedly we find no clear precedent for this in the literature.

An alternative role for this protein is that of the preceedented TE2, TE2s are well described in association with Pks and NRPS modules in bacteria, fungi and plants, and serve to proofread the nascent product on the synthetic module [39]. They are specific for their corresponding ACP [40,41]. Without a functioning TE2, erroneous acyl chains on a Pks module clog the machinery, decreasing product production by up to 90% [38]. If the Pks product is a secondary metabolite, this diminution is not crucial. However, in the case of mycolic acids, this diminution could render the bacterium non-viable.

A role as a TE1 or as a TE2 are both consistent with the putative essentiality of *Rv3802* and the decrease in mycolic acids observed with THL. Significant amino acid similarity between TE1s or TE2s and cutinases is not found by search programs, yet the TE1s, TE2s and cutinases are all α/β-hydrolase fold enzymes with SDH motifs. In *Rv3802*, which otherwise shares little amino acid similarity to the cutinases (~0–40%), the spacing of the SDH is more similar to thioesterses than to cutinases; structure prediction models find structural similarity of *Rv3802* with known cutinases and thioesterases at 100% and 75% precision, respectively (Figure 8) [42].

Though there are multiple lines of evidence for involvement in mycolic acid synthesis, a drawback of our studies is that the data is indirect; further evaluation is underway, but due to complexity will take some time. The genetic location of *Rv3802* and the thioesterase activity of its gene product are not likely to be coincidental, and inhibition by THL further supports this hypothesis. However, an alternative role interacting with host or mycobacterial lipids by *Rv3802* or the other mycobacterial annotated cutinases is a possibility. These enzymes clearly possess PLAs activity, and are able to hydrolyze the mycobacterial phospholipid PIM. If indeed there is a phospholipid carrier involved in mycolic acid synthesis, or if indeed host derived fatty acids can go directly into the FAS-II pathway, PLAs activity may actually be linked to mycolic acid synthesis [7,10].
Conclusions
Our studies suggest *M. tuberculosis* Rv3802, previously annotated as a cutinase, is involved in mycolic acid synthesis. Activity as either a mycolytransferase or a Pks associated thiosterase are logical roles for Rv3802, and are consistent with its genetic location, its discovered thiosterase activity, its inhibition by THL, and the previously published cell wall defects with use of THL as an antmycobacterial compound. However, this evidence is circumstantial and further direct evidence is needed to support or refute such a role for Rv3802.

Materials and Methods

Bacterial strains and culture conditions
*M. smegmatis* mc² 155 (ATCC 700165) was purchased from ATCC. *M. smegmatis* was grown at 37°C in Luria-Bertani (LB) broth with 0.05% Tween® 80®. *Escherichia coli* strains DH5α and Rosetta 2 (DE3)pLysS (Merck) were grown at 37°C in LB broth. Antibiotics (Sigma) were added to media at the following concentrations: Ampicillin 100 μg/mL, Kanamycin 25–50 μg/mL, Chloramphenicol 34 μg/mL.

Cloning Procedures
Standard PCR strategies with Taq DNA polymerase (Invitrogen) were used to amplify the *M. tuberculosis* Rs3802c and *M. smegmatis* SMSEG_1403 genes. PCR amplifications consisted of one cycle of denaturation (95°C, 10 min), followed by 30 cycles of amplification that included denaturation (95°C, 1 min), annealing (65°C, 1 min), and primer extension (72°C, 1 min). The primers used were as follows: 5’-catatggatccgctgacctgcactggatcggc-3’ and 5’-gggccctgctcaaacccgatcag-3’ for *M. smegmatis* SMSEG_1403 gene, and 5’-catatggcgaatcgccgcccagcgcgg-3’ and 5’-gagctcgggtttctcgtgcggctctgactcccagg-3’ for *M. tuberculosis* Rs3802c gene. Primers were designed to provide PCR amplified fragments containing a C-terminal streptavidin tag (bold) and flanking *NdeI* and *SalI* restriction sites (underline). Fragments were cloned into pET23a (Novagen) and colonies for subsequent cloning were confirmed with sequencing. Correct clones were sub cloned into pHET23a (Novagen). This vector was used as a template to make three mutants in *M. tuberculosis* Rs3802c via the QuickChange® II method (Stratagene, 2005245, per manufacturer protocol). Primers used were 5’-cgatggctggtagaagggcggg-3’ and 5’-cggcggcgtcctgctcaacctgcag-3’ for Rv3802 ΔS8175E, 5’-gaaacttttgagCcggatgagcggcggg-3’ and 5’-cctctcggggtgattctgctg-3’ for Rv3802 ΔS866E, and 5’-cttctcggggtgattctgctg-3’ and 5’-gctggctggcggctggctccggcggg-3’ for Rv3802 ΔS876E.

Expression and purification of recombinant proteins

*M. tuberculosis* Rs3802, its mutants, and *M. smegmatis* mc² 155 MSSEG_1403 in E. coli

The constructs in pET23a described above were transformed into electrocompetent Rosetta 2 (DE3)pLysS for expression using the T7 promoter. Cultures were induced with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) for 3 H at 37°C. Following induction, cell pellets were collected and re-suspended in 5 mL Streptactin lysis buffer (50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0 with 1 mg/mL lysozyme and 5 μg/mL DNAse I) per 200 mL original induction culture, and stored at -20°C overnight. Thawed resuspensions with 2% (w/v) n-lauryl sarcosine was mixed for ≥60 min at room temperature until lystate was clear of large clumps. After centrifugation, target protein was purified from lystate using streptactin Superflow resin (IBA, Germany) per company protocol. Protein concentration and absorbance spectra of these aliquots were evaluated at 280 nm on a Nanodrop™ system. All eluted protein samples, if not used immediately, were stored at -70°C. Selected fractions were also analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and either stained or immunoblotted using an anti-streptavidin tag antibody (IBA) at 1:1500 and secondary horseradish peroxidase goat anti-mouse (1:20 K), and visualized with chemiluminescense. Fractions were estimated to be at least 95% pure using SYPRO orange staining (molecular probes S5692, per protocol, sensitive to 5 ng of protein) with phosphoimager detection.

Whole Cell Radiolabeling Experiments

For radiolabeling of whole *M. smegmatis* mc² 155 cells with ¹⁴C, *M. smegmatis* was grown in LB medium supplemented with 0.05% Tween® 80® and, when in log phase, ¹³C-acetate (ARC 0173B) was added at 1 μCi/mL and cells were grown for four additional H. Cells were then pelleted, and extracted with 30 mL CHCl₃:CH₃OH (1:2, v/v) per gram of wet cells. Debris was again pelleted, extract removed, and re-extracted with the same volume of CHCl₃:CH₃OH (2:1, v/v). Extracts were combined and Folch washed. Organic layer was aliquoted and dried down in pre-weighted vials. For mass spectrometry, a parallel experiment was done without ¹⁴C-acetate. To isolate PIM species, 400 μCi/mL and cells were grown for four additional H.

Mass spectrometry of extracted cell wall lipids

Cell wall lipids were isolated as above. Lipids were applied to Silica gel 60A plates (Whatman, 84507-425), and developed in...
CHCl$_3$:CH$_2$OH:NH$_2$OH (80:20:2, v/v/v). Individual bands of lipid were scraped from the plate into columns made from Pasteur pipettes that were fitted with 0.2 mm. Type FG Fluoropore membrane filters (Millipore). The silica gel was rinsed with 1 mL of 2:1 i-C$_5$H$_7$OH/H$_2$O and lipid species were eluted with 1 mL of 2:1 (v/v) CHCl$_3$/CH$_2$OH. Samples were evaporated to dryness, re-dissolved in 2 µL of the same solvent and 0.5 µL was deposited onto a sample stage that had been pre-spotted with 2,5-dihydroxybenzoic acid (DHB) for analysis by matrix assisted laser desorption ionization (MALDI) mass spectrometry. Mass spectra using a laser wavelength of 337 nm were obtained from m/z 100 to 2400 using a QSTAR XL tandem mass spectrometer (Applied Biosystems/MDS Sciex, Thornhill, Ontario, Canada). The mass analyzer configuration of this instrument, mass-resolving quadrupole – collision quadrupole – orthogonal time-of-flight (Q-q-oTOF), allowed both full and collision induced dissociation (CID) spectra to be obtained. PIM species were identified based on molecular weight, interpretation of CID spectra, and comparison of spectra with data found in the literature [43,44].

**Cell-free Assay on M. smegmatis crude cell wall and purified PIM components**

Aliquots of $^{14}$C-radiolabeled cell wall or purified PIM were resuspended at 2 mg/mL in 50 mM Bis-tris, pH 7, with 0.25% Triton®-X 100. 50 µL of this was added to 10–50 µg of enzyme in 50 µL, for a total reaction volume of 100 µL. After 2–5 h, the reaction was quenched with 200 µL of CHCl$_3$:CH$_2$OH:HCl (50:50:0.3, v/v/v) and the organic phase removed and plated under a nitrogen stream on Silica gel 60A˚ plates (Whatman, 4807-425). Plates were developed in CHCl$_3$:CH$_2$OH:NH$_2$OH (80:20:2, v/v/v) and visualized with a phosphoimager (radiolabeled) and/or cupric sulfate or Dittmer-Lester stains (radiolabeled and unla-

**Thioesterase, esterase, and transferase activity of recombinant of M. tuberculosis Rv3802, its mutants, and M. smegmatis mc$^2$155 MSMEG_1403, with and without the inhibitor THL**

Control enzymes included F. solani cutinase, the general esterase Thermomyces lanuginosus (Sigma L-0777) and M. tuberculosis Ag85C (Tuberculosis Research Materials and Vaccine Testing, Colorado State University).

**Nitrophenyl ester assays.** hydrolysis of NP (Sigma, N9876) was used as a surrogate for cutinase activity. Though both M. smegmatis MSMEG_1403 and M. tuberculosis Rv3802 were active in this assay, kinetics was done with only Rv3802. For kinetic calculations, each 200 µL reaction contained 0.01 mg of enzyme (1.53 mM), the path length was 0.8 cm and the extinction coefficient was 15250 M$^{-1}$ cm$^{-1}$ [45]. The NP 5.69 M stock was diluted in 50 mM bis-tris/5% glycerol (pH 7.0) for use in the assays. The final substrate concentration ranged from 1.56 mM–200 mM. Assays were done in 96 well plates, in triplicate, at 37 °C for four h, with proper controls. There were three replicate experiments. Absorbance was read by a plate spectrophotometer at 415 nm at various time points. Prizm 5.0 software were used to determine $K_m$ and $V_{max}$ and these were used to determine the $k_{cat}$ and specificity constant for the esterase activity of Rv3802 and mutants. Up to 10 units of snake venom PLA was also tested and was inactive.

**Thioesterase activity assays.** hydrolysis of P-CoA (Avanti, 870716) and D-CoA, (Sigma, D5269) were used to measure thioesterase activity. When the thioester bond is hydrolyzed, the free sulfur on CoA is attacked by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), which releases a measurable nitrophenyl group, 5-thio-2-nitrobenzoate (TNB$^2$$-$). For kinetic calculations, each 200 µL reaction contained 3 µg of enzyme (461 nm), the path length was 0.8 cm and the extinction coefficient was 11450 M$^{-1}$ cm$^{-1}$ for TNB$^2$ (Sigma). The P-CoA 20 mM stock was diluted in 50 mM bis-tris (pH 7.0) for use in the assays. The DTNB 40 mM stock was diluted in EtOH to give a molar equivalent amount of DTNB to acyl-CoA for the reaction (Increasing DTNB relative to P-CoA did not increase reaction rate, and DTNB did not react with enzyme alone). The final P-CoA concentration ranged from 4.875 µM–2.5 mM, and the final D-CoA concentration ranged from 0.078 to 5.0 mM. Assays were done in 96 well plates, in triplicate, at 37 °C for three h, with proper controls. There were four replicate experiments. Absorbance was read by a plate spectrophotometer at 415 nm at various time points. Prizm 5.0 software were used to determine $K_m$ and $V_{max}$ and these were used to determine the $k_{cat}$ and specificity constant for the thioesterase activity of Rv3802. For radiolabeled $^{14}$C-P-CoA assays, protocol was followed as in $^{14}$C-phospholipids assays above.

**THL inhibition assays.** four concentrations of THL (Sigma, 04139) were selected in order to determine $K_i$ and nature of inhibition of Rv3802, based on pilot experiments: 1.56 µM, 0.78 µM, 0.39 µM, and 0.195 µM. 100% inhibition of activity was seen at 3 µM final concentration. Inhibition assays were performed identically to the thioesterase assays without inhibitor described previously, except that after the addition of THL and before the addition of P-CoA, the assay was incubated at 37°C for 30 min [39]. The Reaction was run for 1–2 h, in duplicate, and four replicates were performed. Changes in $K_m$ and $V_{max}$.
(compared to non-inhibited Rx3802) were determined using Prizm 5.0 software, as were apparent Kₐ and IC₅₀ values. For inhibition of M. smegmatis MSMEG_1403, each 200 µL reaction contained 0.5 µg (0.495 µM); THL concentration was increased to a maximum of 25 µM (a molar excess compared to enzyme of 50:1) in an attempt to detect inhibition.

**Transferase assays.** donors and recipients were paired for various transferase assays to assess whether the purified enzymes could hydrolyze a known substrate and transfer it to an acceptor. Assays included 10–50 µg of either Rx3802 or MSMEG_1403 protein, and were incubated for 1–72 H. Pairings included: 1) 14C-trehalose (0.25 µgCi) with crude cell wall lipids (50 µg) from M. tuberculosis; 2) 14C-P-CoA (0.25 µgCi) as donor with mannose-6-phosphate (1–10 mM) or glycerol-6-phosphate (1 mM) as acceptor with crude cell wall extract (proteins and lipids for unknown cofactors or other acceptors, total 0.1 to 1 mg, made per previously described protocol[27]); 3) same as two with addition of crude cytosolic extract, 4) same as in two and three with addition of 14C-trehalose (0.25 µgCi); 5) same as two and three with addition of 14C-DPPC instead of 14C-P-CoA. As a positive control for the mycolyltransferase assays using 14C-trehalose as an acceptor, Ag85C was used at 10–50 µg per assay.

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**Author Contributions**
Conceived and designed the experiments: SKP RMB. Performed the experiments: SKP RMB JGR. Analyzed the data: SKP RMB JGR. Contributed reagents/materials/analysis tools: SKP RMB MLV. Wrote the paper: SKP.

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