Exposure to a cutinase-like serine esterase triggers rapid lysis of multiple mycobacterial species.

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Running title: Mycobacterial lysis by an esterase

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Capsule:

**Background:** Mycobacteria possess a unique envelope with poorly understood architectural organization of constituent molecules.

**Result:** Exposure of mycobacteria to an esterase that targets trehalose mycolates triggers rapid lysis of bacteria.

**Conclusion:** Trehalose mycolates are on the exposed surface of the envelope with an indispensable structural role.

**Significance:** The study identifies a novel enzyme-based lysis of mycobacteria, with a potential for new anti-tuberculosis applications.

Summary

Mycobacteria are shaped by a thick envelope made of an array of uniquely structured lipids and polysaccharides. However, the spatial organizations of these molecules remain unclear. Here we show that exposure to an esterase from *Mycobacterium smegmatis* (Msmeg\_1529), hydrolyzing the ester linkage of trehalose dimycolate (TDM) \textit{in vitro}, triggers rapid and efficient lysis of *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium marinum*. Exposure to the esterase immediately releases free mycolic acids, while concomitantly depleting trehalose mycolates. Moreover, lysis could be competitively inhibited by an excess of purified TDM and was abolished by a S124A mutation affecting the catalytic activity of the esterase. These findings are consistent with an indispensable structural role of trehalose mycolates in architectural design of the exposed surface of mycobacterial envelope. Importantly, we also demonstrate that the esterase-mediated rapid lysis of *M. tuberculosis* significantly improves its detection in paucibacillary samples.

Introduction

Mycobacteria represent a group of ubiquitous and diverse bacterial species, many of which can establish chronic infections in humans (1). An estimated one-third of the world’s population is infected by *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis (TB) which causes about 1.7 million deaths worldwide every year (2). A distinct taxonomical classification for mycobacteria historically originated from their unique cellular morphology and acid-fastness, both directly attributed to their thick, hydrophobic and atypically structured envelope (3-6). The hydrophobicity of the envelope, and consequently its low permeability to hydrophilic solutes, contribute to a high level of intrinsic drug tolerance in mycobacteria (7). Not surprisingly, the envelope of mycobacteria has long remained a subject of intense investigation, yet its architectural organization remains to be fully understood.

The mycobacterial cell envelope is broadly stratified into a plasma membrane
made of phospholipids, a core cell wall complex of covalently linked mycolyl-arabinogalactan-peptidoglycan (mAGP), and a membrane-like outer layer (6,8,9). The lipid bilayer in the membrane-like outer layer, also referred to as the mycomembrane, is formed by mycolyl chains of mAGP and the hydrocarbon chains of the outermost glycolipids, non-covalently associated with the cell wall core of the envelope (6,9). The outermost lipids, which vary across the mycobacterial species, are extractable to a detectable level without breaching the envelope integrity, although genetic dispensability of only a few of these lipids is determined (10).

The mycolyl esters of trehalose monomycolates (TMM) and trehalose 6,6’ dimycolate (TDM) are among the most conserved and abundant non-covalently associated lipids of the mycobacterial envelope. The nascent mycolyl chains, synthesized in the cytoplasm by fatty acid synthase (FAS) systems I and II, are esterified to trehalose to produce TMM, which is subsequently transported across the membrane by MmpL3 (11,12). The abundant pool of TMM in the envelope is then utilized as a universal mycolyl donor by the secreted mycolyltransferases, antigen 85 complex (Ag85A, Ag85B and Ag85C), towards the constitutive synthesis of mAGP and TDM (11,13-15). In addition, TMM is also used by Ag85 complex to synthesize glucose monomycolate, which like other non-covalently linked mycolyl glycolipids, such as glycerol monomycolate and diarabinodimycolyl glycerol, is produced under specific growth conditions depending on the type of carbon source in the medium (16-18).

The essential role of TMM as a precursor of the core cell wall component makes it an indispensable lipid for mycobacteria, recently validated by the viability loss due to specific inhibition of its MmpL3 transporter (12,19). The dispensability of TDM in the envelope, however, remains unclear, although it can be extracted from the bacilli in petroleum ether without any effect on their viability (20), but the extent of TDM depletion from the ether-treated bacteria remained unclear in these studies. Furthermore, TDM cannot be genetically depleted from mycobacteria due to the functional redundancies in the Ag85A, B and C (13). A mutation in ag85A leads to partial depletion of TDM in M. smegmatis, and inactivation or inhibition of Ag85C in M. tuberculosis leads to partial loss in mycolyl content of mAGP as well as synthesis of TDM (14,21). Moreover, the partial loss in the mycolyl contents of cell wall components in the ag85A mutant could be complemented by expression of Ag85B and Ag85C (14). Although any of the three mycolyltransferases can be inactivated individually without affecting cellular viability, simultaneous inactivation of all three genes has not been possible. It is also noteworthy that their simultaneous intracellular depletion using antisense oligonucleotides severely retards the growth of M. tuberculosis (22).

TDM is studied in M. tuberculosis as one of the most potent immunomodulatory and granulomatogenic surface glycolipids (23,24), although it is highly abundant in at least nine other species of pathogenic and non-pathogenic mycobacteria analyzed so far (10,25,26). Its constitutive abundance in non-pathogenic mycobacterial species raises a possibility that TDM and the other non-covalently linked mycolyl-glycolipids could have crucial structural contributions in the integrity of the mycobacterial envelope. Recently, we identified Msmeq_1529, an M. smegmatis enzyme of the serine esterase superfamily that can hydrolyze purified TDM from various mycobacterial species, including M. tuberculosis in vitro (27). This allowed us to question whether exogenous exposure to the purified recombinant esterase, henceforth called TDMH, may impact the integrity of the mycobacterial envelope. Here we show that exposure to TDMH triggers an immediate release of free mycolic acids (FM) from the non-covalently associated mycolyl-containing glycolipids, ultimately leading to rapid and extensive lysis of pathogenic species, such as M. tuberculosis, M. bovis and M. marinum, as well as to a lesser extent of M. smegmatis and M. avium. While these findings highlight the structural contribution and importance of
mycolyl glycolipids in the outer envelope of mycobacteria, they also open up new possibilities of improved detection and clearance of mycobacterial infections.

**Experimental Procedure**

**Strains and media**
Liquid cultures of *M. tuberculosis* (except mc²7000), *M. bovis* (BCG) and *M. avium* were grown at 37°C either in 7H9OADC or Sauton’s media with 0.05% Tween-80 (Tw). For plate culture 7H11 with OADC (Oleic, Albumin, Dextrose, Catalase) or Sauton’s media agar medium was used. mc²7000 was grown similarly, except its media contained 100 µg/mL of pantothenic acid. For *M. smegmatis*, OADC was replaced with ADC in 7H9 broth or plate culture. *M. marinum* was grown at room temperature in 7H9OADCTw liquid medium or Middlebrook 7H11 agar. *E. coli* (DH5α) was grown at 37°C in LB broth or LB agar.

**Purification and in vitro lytic activity of TDMH**
The open reading frame of TDMH, Msmeg_1529, was cloned in pET21b expression system with His₆ tag, and the protein was purified on Ni-NTA affinity column as previously described (27). Functional activity of each batch of purified TDMH was determined through turbidimetric measurement of bacterial lysis as described previously (28). For lytic activity, cells at OD of 0.5 (log-phase) or OD of 3.0 (stationary phase) were harvested, washed and resuspended in either PBST or other culture media as required. Specified amounts of TDMH were mixed with specified number of bacilli and incubated at 37°C. At regular time intervals, an aliquot was diluted and plated for enumeration. An equal volume of storage buffer was used as a negative control for each experiment. For inhibition of lytic activity of the enzyme, 100µg of either purified TDM (Sigma), purified apolar and polar lipids (27), or purified preparations of mAGP and PIM₂ (27) were dried in the reaction tube and suspended in 100µL PBST by sonication at 55°C for 10 minutes. To the lipid suspension, cells (10⁷ cfu/mL) and 0.8µM TDMH were added and incubated for two days at 37°C prior to enumerating the viable bacilli. For the plate assay, 500µL of 10⁶ cfu/mL log-phase cells was spread on a Sauton’s agar plate and 200µg of purified TDMH were spotted in the center. An equal volume of storage buffer was spotted as a negative control on a separate plate with bacilli.

**ATP release assay**
*M. tuberculosis* mc²7000 was grown to OD of 0.5. Cells were harvested, washed with PBS and diluted to 10⁸ cfu/mL in PBST. 8µM of TDMH was added to 1mL cell suspension and rotated at 37°C. At regular time intervals, 100 µL samples were collected and ATP content was determined by adding 100µL of ENLITEN Luciferase/Luciferin reagent (Promega), and the luminescence was measured using a Monolight 2010 luminometer.

**Lipid Analysis**
mc²7000 cultures (OD of 0.5) were labeled with ¹⁴C-acetate for six hours, washed, resuspended in 250 µL of PBST at a density of 10⁹ cfu/mL, mixed with either 8 µM TDMH or an equal volume of storage buffer at 37°C. At specific time intervals apolar and polar lipids were extracted in petroleum ether as published (27). The extracted lipids equivalent to 5000 cpm from each sample were spotted on a one-dimensional TLC, and developed in either CHCl₃/MeOH (97:3, v/v) for FM analysis, CHCl₃/MeOH/H₂O (90:10:1, v/v/v) TDM analysis, or CHCl₃/MeOH/NH₄OH (80:20:2, v/v/v) for TMM analysis, as described earlier (13,27,29). Endogenously purified ¹⁴C-FM, ¹⁴C-TDM, and ¹⁴C-TMM were used as standards. For 2D-TLC, amounts equivalent to 35,000 counts of each sample were analyzed as described previously (27). For the analysis of FM release in the presence of exogenous TDM and TMM, 100µg of TDM or 60µg of TMM were sonicated in 100µL PBST prior to adding 50µg of TDMH (corresponding to 20-fold molar excess of lipid to the enzyme) to a final volume of 125µL. The lipid-enzyme mixture was incubated for 30 min, prior to
adding it to a 125µL suspension of 14C-labeled *M. tuberculosis* cells at 10⁶ cfu/mL.

**In vitro catalytic activity of TDMH**

14C-TDM or 14C-TMM, equivalent to 100,000 cpm was homogeneously suspended in the assay buffer as described earlier (27). The homogenate was then mixed with either 5µg of TDMH, 5µg of TDMH (S124A) or an equal volume of storage buffer, and incubated at 37°C for two hours. The lipids present in the reaction mixture were then sequentially extracted with an equal volume of petroleum ether followed by dichloromethane. The organic layer was dried and analyzed on TLC developed in CHCl₃/MeOH (97:3) (27).

**Nucleic acids detection and RT-PCR**

For determining nucleic acids, 10µL of 10⁷ cfu/mL of *M. tuberculosis* (Erdman) was mixed with either 1µL of 13µg TDMH, or 1µL of storage buffer in PBST supplemented with 26mM L-asparagine (PBSTA), and incubated at 37°C. At various time intervals reactions were heat-inactivated at 80°C for 20 minutes, centrifuged at 14000 rpm for 30 sec., and 1µL of the reaction was place in a Nanophotometer to measure the NA contents. For RT-PCR, in a 10µL reaction 10⁴ cfu/mL bacilli of *M. tuberculosis* (Erdman) were mixed with either 13µg of TDMH, or equivalent volume of storage buffer in PBSTA and incubated for 30 minutes at 37°C, then heat-inactivated at 80°C for 20 minutes. After centrifugation at 14000 rpm for 30sec, 1 µL of the supernatant from the treated mixture was directly added as a template to the molecular beacon based RT-PCR described previously (30), with 500nM each of the *M. tuberculosis* 16S rRNA-specific forward and reverse primers, 200nM of molecular beacon and 5 µL of 2X RT-PCR master mix (Applied Biosystems). The amplification conditions were: 95°C for 10 minutes, followed by 40 cycles of: 95°C for 30 seconds, 58°C for 60 seconds and 72°C for 30 seconds in Applied Biosystems instrument (StepOnePlus RT-PCR System). The forward and reverse primer sequences were 5’-GAGATACTCGAGTGGCGAAC-3’ and 5’-GGCCGGCTACCCGTCGTC-3’ respectively, and the molecular beacon consisted of a5’-fluorescein-GCGCCCAGCCCGGCCTATCAGCTTGTTGGTGGCCGC-dabcyl-3’. The statistical significance between treated and untreated samples was determined by a random intercept logistic regression model.

**Electron microscopy: sample preparation and analysis**

mc²7000 cells (10⁹ cfu/mL) mixed with 8µM of TDMH in PBST, harvested after a 12-hour incubation, put on ice, and transferred to Leica EM AFS (Leica) for a 5-day solvent substitution. Briefly, frozen samples were warmed up from -196°C to -90°C over three days in a precooled (-90°C) 1% OsO₄ and 0.1% Uranyl Acetate mixture dissolved in acetone. Samples were then gradually warmed up to room temperature over 18 hours and subsequently rinsed in acetone for further resin infiltration and embedding. Ultrathin sections (65 nm) were cut with a Reichart Ultracut and laid on 300 mesh carbon coated EM grids. The thin sections were post-stained with 2% uranyl acetate in methanol for 10 minutes, followed by Reynold’s lead citrate (32) for 7 minutes, and were examined with a Tecnai F20 electron microscope (FEI) equipped with a 4k x 4k camera (Gatan).

**Results**

Exposure to TDMH leads to a rapid loss of *M. tuberculosis* viability

Approximately 10⁶ cfu/mL of *M. tuberculosis* H37Rv were incubated with increasing concentrations of the purified recombinant TDMH for 24 hours in PBST. Figure 1A shows that the viability of the population was reduced by at least 100-fold after exposure to 0.8µM or higher concentrations of the enzyme (Fig 1A). At a 10-fold excess concentration (8µM), the enzyme could reduce the viability of 10⁶cfu/mL of bacilli in less than two hours, but higher density (10⁷cfu/mL) required longer periods exposure (Fig 1B).
We next evaluated TDMH activity in more complex chemical environments in which mycobacteria are routinely cultured or studied in the laboratory. TDMH activity varied widely from the highest in Sauton’s media to the lowest in 7H9/OADC and DMEM-FCS (Fig 1C), although no protein degradation was observed under these conditions (data not shown). Removal of the albumin-based supplement (OADC) restored TDMH activity in both 7H9 and DMEM base, but not to the levels observed in Sauton’s broth medium (Fig 1C). Because the activity in Sauton’s media was even more pronounced than in PBS, we hypothesized that one or more components of Sauton’s medium could potentially enhance the enzyme activity. Glycine has been previously found to sensitize mycobacteria against envelope-targeting agents like lysozyme (33). Although it is not clear how glycine could destabilize the cell envelope of mycobacteria, D-amino acids have been recently discovered to depolymerize amyloid-like protein fibers on the surface of gram-positive and gram-negative bacterial envelope (34). We, therefore, reasoned that high concentration (26mM) of L-Asparagine (L-Asn) in Sauton’s media could possibly sensitize mycobacteria to TDMH. Indeed, we found that an equivalent amount of L-Asn increases TDMH efficacy in PBST by 100-fold (Fig 1D). To exclude the possible interference of Tween-80, known to influence the properties of the mycobacterial envelope (35), we tested the activity of the TDMH in PBS without Tween-80 and found no significant change in the lytic activity of the enzyme (Fig 1E). We next assayed the activity of the TDMH directly on a lawn of *M. tuberculosis* on a detergent-free Sauton’s media agar plate. An unambiguous zone of clearance around the spotted area was observed after three weeks of incubation at 37°C (Fig 1F). Overall, these results indicate that exposure to TDMH causes a very rapid and efficient loss of *M. tuberculosis* viability in diverse in vitro conditions.

Viability loss is due to compromised envelope integrity and cellular lysis. The most likely explanation for loss of viability in TDMH-exposed bacteria is a breach of the envelope integrity, and a subsequent bacterial lysis caused by the hydrolysis of target molecules. Lysis was evidenced by the clearance of a turbid suspension of *M. tuberculosis* after exposure to 8µM of TDMH for 48 hours (Fig 2A). Lysis was further confirmed by the release of ATP from TDMH-exposed *M. tuberculosis* mc27000 (Fig 2B). The timing of ATP release in Figure 2B was also consistent with the loss of cell viability at this density (Fig 1B). We examined the integrity of the TDMH-treated bacteria at an ultra-structural level by high-pressure freezing and freeze-substitution electron microscopy. As expected, a distinct multi-layered architecture of the envelope, including an outer layer, was observed in the untreated bacteria (Fig 2C, i-iii). In the TDMH-exposed bacilli, the outer layer was visibly ablated at several places (Fig 2C, iv-viii). We could also capture cells that were either in the process of losing the cytoplasmic content during lysis (Fig 2C, v to vii), or had completely lost the cytosolic content and the outer cell envelope (Fig 2C, viii). The heterogeneous state of TDMH treated bacilli and the partial loss of the envelope integrity can be attributed to the high density (10⁹ cfu/mL) of bacilli exposed to sub-saturating concentration (8µM) of the enzyme as indicated by the slower lysis (Fig 1B). These observations together are fully consistent with the idea that exposure to TDMH breaches the integrity of the outer layer of cell envelope, thereby triggering bacterial lysis. Henceforth, activity of each TDMH preparation was determined by turbidimetric measurement of cell lysis as described earlier for a bacteriophage lysin (28). TDMH-dependent lysis through exogenous disruption of the envelope implies that the anti-mycobacterial activity of the enzyme will be non-responsive to the physiological state of the target bacilli. This was indeed supported by a comparable susceptibility profile of exponentially growing and stationary phase bacilli to TDMH exposure (Fig 2D).

We further confirmed the relationship between TDMH activity and bacterial lysis by
engineering a point mutation in the catalytic site of TDMH, and testing the lytic activity of the mutant enzyme. Cutinase-like serine esterases possess a highly conserved catalytic triad of serine (in a GXSXG motif), aspartate and histidine (27). A catalytically inactive TDMH mutant was generated by replacing the catalytic Ser121 by an Ala residue. As shown in Figure 2E, TDMH(S124A) clearly failed to hydrolyze TDM (Fig 2E). Importantly, exposure of M. tuberculosis to the mutant enzyme failed to lyse the culture (Fig 2F). It can therefore be inferred that exposure to the TDM hydrolase activity causes envelope rupture and mycobacterial lysis.

Depletion of TMM and TDM during early phase of TDMH exposure

Since TDMH hydrolyzes TDM \textit{in vitro} to release FM (27), we next analyzed the levels of FM and TDM in enzyme-exposed \textit{M. tuberculosis} cultures. To this aim, a high bacterial density (10^9 cfu/mL) was used to obtain sufficient lipids in a small volume with an effective amount (8\muM) of TDMH. Because lysis is slowed at this high cell density (Fig 1B), the reactions were followed for an extended period of six days. The relative levels of FM in TDMH-bacteria mixture progressively increased, while it remained almost unchanged in buffer-treated cells (Fig 3A and 3B). After 48-hour exposure the FM levels were over twice the amounts present at the beginning of the exposure (Fig. 3E). Interestingly, while the level of FM started to accumulate very early, within the first hour of treatment, the depletion of TDM was essentially apparent only after 48-hour exposure, when its level reduced over 50\% to the amount seen at the beginning (Fig 3A, B and E). The delayed depletion of TDM suggests that FM is generated at the expense of another mycolyl ester, which is either directly targeted by the enzyme, or indirectly utilized by the cell to replenish TDM. Besides TDM, mAGP and TMM are the two most dominant esters of mycolic acids within the mycobacterial cell wall. No significant change in the mycolyl content of mAGP was observed in TDMH-exposed cells during FM release (Fig 3C), consistent with the lack of \textit{in vitro} hydrolytic activity of the esterase against purified mAGP (27). We next examined whether FM was released at the expense of the TDM precursor, TMM. Figure 3D shows that the levels of TMM in TDMH-exposed cells indeed started to progressively decrease within one hour of exposure, concomitant with the release of FM seen in Figure 3B (Fig 3D).

Since no loss of viability was observed during the first two hours of exposure under these conditions (Fig 1B), TMM depletion and FM release are very likely to be the pre-lysis events, suggesting that non-covalently cell wall-associated mycolyl esters are among the first targets of the enzymes. We also addressed whether any other lipids besides TMM are depleted during the early phase of TDMH exposure. Therefore, a comprehensive analysis was performed on both polar and apolar lipids from 10^9 cfu/mL of \textit{14}C-labeled \textit{M. tuberculosis} treated with either buffer or 8\muM of TDMH for two hours. Besides TMM depletion, and concomitant release of FM, there was no significant depletion in any of the polar or apolar lipids in the TDMH-treated cells (Fig 4A). However, we noted elevated levels of phosphatidyl inositol (PI) and an unknown polar lipid in TDMH-exposed bacilli (Fig 4A). Because there was no obvious depletion of any other lipids to account for the elevated levels of these polar lipids, it is likely that these are produced through anabolic pathways as an early cellular response to TDMH exposure. Taken together, these results suggest that TMM is the major lipid that is significantly depleted during the first two hours of TDMH exposure. It is, however, noteworthy that in contrast to TDM, purified TMM could not be hydrolyzed by TDMH \textit{in vitro} (Fig 4B). This is rather surprising and difficult to reconcile with the \textit{in vivo} effect of TDHM on TMM production. One possibility could be that TMM depletion during early exposure to the enzyme is countered by a response that induces its synthesis by utilizing TMM. In this scenario TDM would be depleted earlier if heat-inactivated cells were exposed to the esterase. Indeed we observed that the levels of TDM decreased to about 50\% within the first one hour when heat-inactivated cells were
exposed to the enzyme (Fig 3F). In addition, we also observed a slower and less pronounced depletion of TMM in the TDMH-exposed heat-killed bacilli (Fig 3F). However, depletion of TMM remained significant in heat-killed cells, a decrease by about 40% within first one hour. No other glycolipids were altered in the enzyme-exposed heat-killed cells during the first two hours of exposure (Fig 3G).

To further substantiate the impact of the TDMH on cellular TDM, we next analyzed the effect of exogenous addition of purified TDM on the enzyme-exposed M. tuberculosis. Addition of 20-fold molar excess of purified TDM with respect to TDMH reduced the release of FM from the bacilli by about 30% within two hours of exposure (Fig 5A). However, a comparable molar excess of exogenous TMM had no effect on FM release (Fig 5A), consistent with the inability of the enzyme to hydrolyze purified TMM in vitro (Fig 4B). Similarly, a 200-fold molar excess of TDM with respect to TDMH could abrogate the lytic activity of the enzyme (Fig 5B). The inhibition in the presence of equivalent weight of a TDM containing apolar lipid fraction was partial (Fig 5B). However, neither polar lipid, nor purified mAGP and PIM, a major polar lipid of the cell envelope (Fig 4A), had any effect on the lysis (Fig 5B). The failure of mAGP to inhibit the lytic activity is consistent with; a) the lack of any apparent loss of its mycolyl content in the exposed cell (Fig 3C), and b) the inability of the enzyme to hydrolyze mAGP (27). Overall, inhibition of FM release and bacterial lysis by exogenous TDM, and early depletion of TDM from the heat-killed bacilli during the esterase exposure support the idea that TDM is surface-exposed where it can directly interact with the exogenous enzyme. Requirement of a large molar excess of purified TDM for an apparent inhibition TDMH activity on M. tuberculosis suggests that the conformation of the lipid in the envelope matrix could be more sensitive to the esterase mediated hydrolysis than those in the aqueous form.

It is noteworthy that the extractability of TDM in petroleum ether without the loss of bacterial viability gives rise to a notion of its dispensability in mycobacteria (20,36). We therefore determined TDM levels in: a) petroleum ether extracts, b) the residual lipids obtained by re-extraction of petroleum ether treated cells in CHCl3/MeOH (2:1, v/v), and c) total lipid extracts from cells in CHCl3/MeOH (2:1, v/v). To exclude the bias in lipid extraction, we measured the amount of total lipids in the three extracts. The amounts of lipids in conditions a) and b) were nearly equal to those obtained in condition c) (Fig 5C). Analysis of TDM on radio-TLCs in three independent extractions revealed that the ether-extracted fraction contained an average of about 5% of the TDM present in the total lipid mixture. Over 60% of the glycolipid were re-extracted along with the residual lipids of the ether-treated bacteria (Fig 5D), indicating that only a limited amount of TDM is extractable in petroleum ether and that this level of depletion has no effect on mycobacterial viability.

**TDMH exposure is detrimental to other mycobacterial species**

Because TDM and TMM are highly conserved glycoconjugates of mycobacteria (25,26), the question arises whether the exogenous TDMH exposure could be similarly detrimental to other mycobacterial species. We tested the activity of TDMH against four widely studied mycobacterial species, M. bovis (BCG), M. avium, M. marinum, and M. smegmatis - representatives of M. tuberculosis complex (MTC) as well as non-tuberculous mycobacteria (NTM). Although loss of viability was observed in all four of them, M. smegmatis and M. avium were significantly more tolerant than the other species (Fig 6A). Because mycolyl esters of trehalose are unique lipids of mycobacteria, TDMH exposure expectedly had no effect on E. coli (Fig 6A). The decreased sensitivity of M. smegmatis and M. avium suggests that their trehalose mycolates are either less exposed or recognized with a lower affinity by the enzyme. The tolerance observed in M. smegmatis could possibly be attributed to the fact that the enzyme is physiologically expressed by this species, and therefore may
have evolved a mechanism to control the activity on its own envelope. In addition, the TDMH tolerance could also originate in the distinct species of mycolates conjugated to trehalose in M. smegmatis, for instance, presence of shorter chain α', and absence of methoxy- and keto- species (11). Interestingly, M. marinum appears very sensitive to TDMH (Fig 6A). The failure of TDMH(S124A) to lyse M. marinum cells (Fig 6B) suggests that depletion of trehalose mycolates could be the likely trigger of lysis in this species as well.

**TDMH facilitates M. tuberculosis detection at low density**

A rapid and efficient lysis of TDMH exposed M. tuberculosis and subsequent release of nucleic acids (NA) offers a unique opportunity to use this enzyme for achieving more sensitive detection in a nucleic acid amplification assay for TB diagnosis. NA content in the supernatant of TDMH exposed bacilli indeed started to increase within 30 minutes of exposure, and peaked at around 60 minutes (Fig 7A). A prolonged exposure however decreased the NA content, presumably because of the activity of cellular nucleases released in the lysates. Interestingly, a cell suspension at this density treated with the storage buffer had low but detectable amounts of NA that remained unchanged during the exposure time, indicating a spontaneous lysis of bacterial at low frequency during heating (Fig 7A). We next performed a molecular beacon based 40-cycle Real time-PCR reaction (30), to detect NA corresponding to 16S-rRNA in samples with less than 10 bacilli. Out of 45 independent amplification reactions in paucibacillary samples, diluted in triplicate from 15-independent broth cultures, NA could be detected in only seven reactions without TDMH treatment (7B and 7C). However, treatment of samples with TDMH prior to the amplification reaction facilitated the NA detection in 37 samples; highly significant (p < 0.0001) improvement in the numbers of positive detection (Fig 7C). Low frequency of NA detection in some of the untreated samples is consistent with Poisson distribution pattern of dilution in which some dilutions have larger enough bacilli to generate sufficient amount of template due to autolysis during heating.

**Discussion**

In this study, we report an unusual and unexpected consequence of mycobacterial lysis upon exposure to a cutinase-like serine esterase. The enzyme can hydrolyze purified TDM in vitro, and sequentially deplete TMM and TDM from the envelope of the exposed cells. The early depletion of TMM and TDM indicate that these lipids are both exposed on bacterial surface and therefore are among the first molecules encountered by the enzyme. Loss of trehalose mycolates could itself cause osmotic lysis and/or create openings for the enzyme to access the inner components and breach envelope integrity. Either of these situations therefore reveals a structural role of the glycolipids in maintaining the integrity of the mycobacterial envelope.

Involvement of TMM is a more complex scenario to imagine because of the dynamic range of intermediate conformations it could assume during synthesis of terminal mycolyl esters, although a molecular subspecies of the glycolipid with a dedicated structural role cannot be ruled out. The role of TDM, however, is highly plausible because of its large abundance and its stable terminal structure as an end-product. Given that the outermost layer of the envelope assumes a membrane-like configuration (9), TDM could likely be organized in a bilayer configuration constituting the outer leaflet of the mycomembrane, with trehalose as the polar head group at the environmental interface and mycolic acids forming the hydrophobic tail. The 60-90-carbon mycolic acid could either be in an extended conformation to span the entire thickness (~8nm) of the mycomembrane or in folded conformation to fit into the outer leaflet of the bilayer (8,9). The folded mycolic acid chain, presumably formed through interaction of the keto or methoxy groups with the lipid head group (8) is the likely conformation. This is extrapolated from the biophysical analysis of mycolic acid monolayers as well as from the visual evidence of hydrophobic interface in the cryo-electron microscopy of the outer membrane (9,37).
The likely integration of TDM in the outer membrane, its limited extractability from the intact envelope and disintegration of the envelope upon its depletion together raise a strong possibility that TDM, and therefore the outer membrane, could be indispensable for the overall integrity of mycobacteria. Moreover, this is consistent with the cryo-EM visualization of dissolved inner membrane in the region where the outer membrane lipids are removed by detergent extraction (9,38). The indispensability of TDM is also suggested by growth inhibition of M. tuberculosis in the presence of a chemical inhibitor of Ag-85C that reduces the level of TDM without affecting the levels of mAGP and TMM (21). Given the detrimental consequences of TDM exposure, mycobacteria must have a mechanism to regulate its physiological activity. Regulation of TDMH is indeed manifested by a growth-phase dependent induction of its activity towards production of free mycolic acids during biofilm maturation of M. smegmatis (27,39). Furthermore, delayed depletion of TDM upon TDMH exposure also suggests a two-tier regulatory mechanism in which replenishment of the substrate could potentially salvage a dysfunctional regulation of tdmh expression. Although TDM hydrolase in other mycobacteria remains to be discovered, antibodies against some of the cutinase-like esterases, including the closest homologue of TDMH (Rv3452), are produced during active infection of M. tuberculosis in humans, indicating their expression during TB pathogenesis (40).

In summary, TDMH solves a long-standing challenge of exogenously breaching the envelope of mycobacteria. The improved frequency of positive detection upon TDMH treatment of samples with small number of bacilli highlights the potential of the enzyme in elevating the sensitivity of TB diagnosis, particularly in paucibacillary infections.

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List of abbreviations:
DMEM-FCS, Dubelco’s Modified Eagle Medium with Fetal Calf Serum; FM, free mycolic acids; OADC, Oleic acid Albumin Dextrose Catalase; Tw, Tween-80; TDM, trehalose dimycolate; TMM, Trehalose monomycolate; TDMH, TDM hydrolase; TB, tuberculosis; mAGP, mycolyl arabinogalactan; NA, nucleic acids; PBST, Phosphate-buffer saline with Tween-80; PBSTA, Phosphate-buffer saline with Tween-80 and asparagine, PIM phosphatidyl inositol myo-mannoside; TLC, thin-layer chromatography.

References


**Figure Legends**

**Figure 1. Loss of *M. tuberculosis* viability upon TDMH exposure.**

A. Viability of 10^6 cfu/mL of bacilli after exposure to various concentrations of TDMH for 24 hours in PBST. In the control (0 µM) experiment an equivalent volume of storage buffer was used. B. Exposure of various densities of *M. tuberculosis* (mc^27000) to 8µM of TDMH over 72-hour period. C. Viability of bacilli after TDMH exposure in growth media for mycobacteria (Sauton’s media and 7H9 with or without OADC), or for macrophage (DMEM with and without serum). PBST was used as a control. D. Effects of equivalent concentration of constituent amino acid of Sauton’s media (26 mM Asparagine, L-Asn) on the antimycobacterial activity of TDMH in PBST. E. Lytic activity of TDMH against *M. tuberculosis* in detergent-free PBS after 2-day exposure. F. Sauton’s media agar plates containing a lawn of *M. tuberculosis* bacilli with either storage buffer or 200 µg of TDMH spotted in the center, and incubated for 3 weeks at 37°C. The error bars represent the standard errors of three independent experiments.

**Figure 2. TDMH exposure causes lysis of *M. tuberculosis*.**

A. Clearance of *M. tuberculosis* (H37Rv) suspension (10^8 cfu/mL) after 48 hours in PBST containing 8µM of TDMH. In the control, an equal volume of storage buffer was added in the cell suspension. B. Luciferine-luciferase based measurement of ATP released in an *M. tuberculosis* (mc^27000) suspension (10^8 cfu/mL) exposed to 8µM TDMH (also see Supplementary Fig. 1). As a negative control ATP was measured in culture exposed to storage buffer. C. High-pressure freezing and freeze-substitution electron microscopy of 10^9 cfu/mL of *M. tuberculosis* (mc^27000) before (i-iii), and after 12-hour exposure to 8µM TDMH (iv-viii). Images in i-iii are independent fields showing low magnification of intact cells (i), and high magnification of their cross sections before TDMH exposure. A close-up view of the envelope layers is shown in the inset of image iii. The cell inner membrane and outer layer are marked as IM and OL. Images in iv-viii are independent fields showing various stages of lysing bacilli in a TDMH-exposed population. Bacteria shown are: (iv) with a lesion in the outer layer (marked with arrow head); (v) with a distorted cytoplasm, presumably moments before the cytoplasm ejection due to the membrane lesion; (vi and vii) in the act of releasing cytoplasmatic contents and; (viii) completely devoid of cytoplasm. Arrows point to the lesion sites. Scale bars are 100nm for (ii-viii), 1µm for (i). D. Exposure of log-phase...
(7-day old with OD 0.5) and stationary phase (35-day old with OD 3.0) cultures of H37Rv to 8µM TDMH. E. Loss of in vitro hydrolytic activity against TDM in the catalytic serine mutant of TDMH (S124A). F. Viability of 10^7 cfu/mL suspension of M. tuberculosis (H37Rv) exposed to 8µM TDMH (S124A). The error bars represent the standard errors of three independent experiments.

Figure 3. TDMH exposure triggers early release of FM and concomitant depletion of TMM.
A. Radio-thin layer chromatography (radio-TLC) of the lipids in cell-TDMH mixture (marked as T) incubated for specified hours (marked under each lane). Purified ^14C-FM and ^14C-TDM were loaded as markers. In the parallel control experiment, the cells were mixed with the storage buffer (marked as B) and processed similarly. B. Radio-TLC of lipids from the cells exposed to TDMH or storage buffer incubated for shorter time (marked under each lane). Purified ^14C-TDM and ^14C-FM were loaded as markers. TLCs in panels A and B were resolved in CHCl_3/MeOH/H_2O (90:10:1, v/v/v). C. Methyl esters of mycolic acids extracted from the insoluble mAGP fractions of M. tuberculosis treated with either buffer or TDMH for two and six hours. D. Radio-TLC of the lipids from TDMH and buffer exposed cells developed in a polar solvent (CHCl_3/MeOH/NH_4OH (80:20:2, v/v/v) to resolve TMM and TDM. Purified TMM and TDM were used as markers (M). E. Relative change in the levels of TMM, TDM and FM over 48-hour exposure in buffer and TDMH exposed M. tuberculosis. The ratio between 0 (t_0) and 48-hour exposure were obtained from densitometric analysis of TLCs. The error bars denote standard error in the values from three independent experiments. F. Relative change in the levels of TMM and TDM in normal and heat-killed M. tuberculosis exposed to TDMH over the first six hours of exposure. The value at each time point, obtained from densitometric analysis of TLCs, represents the percentage of the lipid relative to t_0 (0-hour). The error bars denote standard error from two and three independent experiments for normal and heat-treated cells, respectively. For all the experiments in panel G. TLC of apolar (developed in CHCl_3/MeOH, 96:4 v/v) and polar glycolipids (developed in CHCl_3/MeOH/H_2O, 75:25:4 v/v/v) of heat-killed bacilli exposed to TDMH for two hours. The major apolar and polar glycolipids, phenolic glycolipids (PGL) and phosphatidylinositol mannosides (PIMs) are marked. For experiments in panels A-G, 10^9 cfu/mL of M. tuberculosis were exposed to 8µM of TDMH.

Figure 4. A. Two-dimensional radio-TLC of total apolar and polar lipids equivalent to 35000cpm extracted from cells treated with either buffer or TDMH for two hours. Apolar lipids were resolved in solvent systems A, C and D, polar lipids were resolved in solvent system E described previously (39). Positions of PDIM (Phthiocerol dimycocerosic acid), PIMs (phosphatidylinositol mannosides), PI (phosphatidylinositol ethanolamine (PE), DPG (diphosphatidylglycerol) are marked based on published reference (29). Positions of TMM, TDM and FM are marked based on the migratory pattern of purified reference standards. B. Radio-TLC showing FM release when ^14C-TDM, but not ^14C-TMM, is incubated with 5µg of TDMH.

Figure 5. TDM is an early target of TDMH. A. Quantitative analysis of relative change in FM levels in M. tuberculosis-TDMH mixture with respect to the buffer-exposed control, as described in panel A. Error bar indicates standard error of the densitometric data obtained from three independent experiments. B. Lytic activity of 0.8µM TDMH against M. tuberculosis (H37Rv) in the presence of 100 µg of either purified TDM, apolar lipids (aPL), polar lipids (pL), PIMs, or the mAGP complex. Percentage viability in each condition was calculated using buffer treated cells as reference (100%). C. Total radio lipids (counts per minutes) extracted from 100mg of ^14C-labeled cells in either three sequential cycles of sonication in 2:1 CHCl_3/MeOH (total), or petroleum ether (PE extractable) followed by re-extraction through three cycles in 2:1 CHCl_3/MeOH (residual). D. Percentage of TDM in ether extracted and residual lipids with...
respect to the total lipids. The values were determined by calculating the total number of TDM equivalent pixels in each of the samples obtained through densitometric analysis of radio-TLC. The error bars represent standard errors of three independent experiments.

Figure 6. Sensitivity of various mycobacterial species to TDMH. A. Lytic activity of 8µM TDMH against 10^7cfu/mL of four other mycobacterial species and E. coli. The percentage survival reflects the ratio of the number of viable bacilli before and after the 48-hour exposure. B. Effect on the viability of 10^7cfu/mL suspension of M. marinum upon exposure to TDMH (S124A) mutant-equivalent to 8µM of the wild-type TDMH. The error bars in panel A and B represent standard errors of three independent experiments.

Figure 7. TDMH-treatment of M. tuberculosis releases nucleic acids and facilitates sensitive detection by RT-PCR. A. Nucleic acids (NA) present in the TDMH-cell mixture measured at various time intervals. Equal volume of enzyme storage buffer was added in the cell suspension as a negative control. The error bars represent standard errors of three independent experiments. B. Detection of M. tuberculosis nucleic acid (NA) corresponding to 16S-rRNA in molecular beacon based RT-PCR reaction RT-PCR performed on sample with approximately 10 bacilli either treated with TDMH or storage buffer control. Controls with genomic DNA of M. tuberculosis as template (Template control) or without any template (NTC) contain equivalent amounts of TDMH. C. A summary table of threshold cycles at which signal was detected in 45 independent RT-PCR reactions from buffer and TDMH treated bacilli, (--) denotes no detectable signal. 15 independent dilutions (D1-D15) from equivalent number of broth cultures were treated in triplicates (R1-R3) with buffer or TDMH. Statistical significance between buffer and TDMH treated samples was calculated by random effect logistic regression.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Exposure to a cutinase-like serine esterase triggers rapid lysis of multiple mycobacterial species.
Yong Yang, Alexandra Bhati, Danxia Ke, Mercedes Gonzalez-Juarrero, Anne Lenaerts, Laurent Kremer, Yann Guerardel, Peijun Zhang and Anil K. Ojha

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