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

Yong Yang, Alexandra Bhatti, Danxia Ke, Mercedes Gonzalez-Juarrero, Anne Lenaerts, Laurent Kremer, Yann Guerardel, Peijun Zhang, Anil K. Ojha

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 1, pp. 382–392, January 4, 2013

Exposure to a Cutinase-like Serine Esterase Triggers Rapid Lysis of Multiple Mycobacterial Species*

Received for publication, September 14, 2012, and in revised form, November 15, 2012 Published, JBC Papers in Press, November 15, 2012, DOI 10.1074/jbc.M112.419754

Yong Yang[‡], Alexandra Bhatti[‡], Danxia Ke[§], Mercedes Gonzalez-Juarrero[¶], Anne Lenaerts[¶], Laurent Kremer[‖], Yann Guerardel[‡‡], Peijun Zhang[§], and Anil K. Ojha[‡1]**

*From the Departments of [‡]Infectious Diseases and Microbiology and [§]Structure Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, the [¶]Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado 80523-4629, the [‖]Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, CNRS UMR 5235 Université Montpellier 2, Montpellier Cedex 05, France, **INSERM, Dynamique des Interactions Membranaires Normales et Pathologiques, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France, and [‡‡]CNRS, UMR 8576, F-596 Villeneuve d'Ascq, France*

Background: Mycobacteria possess a unique envelope with poorly understood architectural organization of constituent molecules.
Results: Exposure to an esterase that targets trehalose mycolates triggers rapid lysis of mycobacteria.
Conclusion: Trehalose mycolates are on the exposed surface of mycobacterial 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.

Mycobacteria are shaped by a thick envelope made of an array of uniquely structured lipids and polysaccharides. However, the spatial organization of these molecules remains unclear. Here, we show that exposure to an esterase from *Mycobacterium smegmatis* (Msmeg_1529), hydrolyzing the ester linkage of trehalose dimycolate *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 trehalose dimycolate 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 the architectural design of the exposed surface of the mycobacterial envelope. Importantly, we also demonstrate that the esterase-mediated rapid lysis of *M. tuberculosis* significantly improves its detection in paucibacillary samples.

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),[2] 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 mycomembrane, is formed by mycolyl chains of mAGP and hydrocarbon chains of the outermost glycolipids, noncovalently associated with the cell wall core of the envelope (6, 9). The outermost lipids, which vary across mycobacterial species, are extractable to a detectable level without breaching envelope integrity, although genetic dispensability of only a few of these lipids is determined (10).

The mycolyl esters of trehalose, trehalose monomycolates (TMM) and trehalose 6,6′-dimycolate (TDM) are among the most conserved and abundant noncovalently associated lipids of the mycobacterial envelope. The nascent mycolyl chains, synthesized in the cytoplasm by fatty-acid synthase 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 secreted mycolyltransferases, antigen 85 complex (Ag85A, Ag85B, and Ag85C), toward the constitutive synthesis of mAGP and TDM (11, 13–15). In addition, TMM is also used by the Ag85 complex to synthesize glucose monomycolate, which like other noncovalently linked mycolyl glycolipids, such as glycerol monomycolate and diarabino-dimycolyl glycerol, is produced under specific growth

* This work was supported, in whole or in part, by National Institutes of Health Grants AI079288 (to A. O.) and GM085043 (to P. Z.).
[1] To whom correspondence should be addressed. E-mail: ano7@pitt.edu.
[2] The abbreviations used are: TB, tuberculosis; FM, free mycolic acid; OADC, oleic acid/albumin/dextrose/catalase; TDM, trehalose dimycolate; TMM, trehalose monomycolate; TDMH, TDM hydrolase; mAGP, mycolyl arabinogalactan; NA, nucleic acid; PIM, phosphatidylinositol *myo*-mannoside.

conditions depending on the type of carbon source in the medium (16–18).

The essential role of TMM as a precursor of mAGP, the core cell wall component, makes it an indispensable lipid for mycobacteria, as recently validated by the viability loss due to specific inhibition of its MmpL3 transporter (12, 19). 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, Ag85B, and Ag85C (13). A mutation in *ag85A* leads to partial depletion of TDM in *Mycobacterium smegmatis,* and inactivation or inhibition of Ag85C in *M. tuberculosis* leads to partial loss in mycolyl content of mAGP as well as the 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 nonpathogenic mycobacteria analyzed so far (10, 25, 26). Its constitutive abundance in nonpathogenic mycobacterial species raises the possibility that TDM and other noncovalently linked mycolyl-glycolipids could have crucial structural contributions in the integrity of the mycobacterial envelope. Recently, we identified Msmeg_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 noncovalently associated mycolyl-containing glycolipids, ultimately leading to rapid and extensive lysis of pathogenic species, such as *M. tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium marinum*, as well as to a lesser extent of *M. smegmatis* and *Mycobacterium avium*. Although 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 PROCEDURES

Strains and Media—Liquid cultures of *M. tuberculosis* (except mc^{2}7000), *M. bovis* (BCG), and *M. avium* were grown at 37 °C either in 7H90 ADC or Sauton's media with 0.05% Tween 80. For plate culture, 7H11 with OADC or Sauton's media agar was used. mc^{2}7000 was grown similarly, except its media contained 100 μg/ml pantothenic acid. For *M. smegmatis,* OADC was replaced with ADC in 7H9 broth or plate culture. *M. marinum* was grown at room temperature in 7H9OADC Tween 80 liquid medium or 7H11 OADC agar. *Escherichia 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 His_6 tag, and the protein was purified on nickel-nitrilotriacetic acid affinity column as described previously (27). Functional activity of each batch of purified TDMH was determined through turbidometric 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 phosphate-buffered saline with Tween 80 (PBST) or other culture media as required. Specified amounts of TDMH were mixed with the 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, 100 μg of either purified TDM (Sigma), purified apolar and polar lipids (27), or purified preparations of mAGP and PIM_2 (27) were dried in the reaction tube and suspended in 100 μl of PBST by sonication at 55 °C for 10 min. To the lipid suspension, cells (10^7 cfu/ml) and 0.8 μM TDMH were added and incubated for 2 days at 37 °C prior to enumerating the viable bacilli. For the plate assay, 500 μl of 10^6 cfu/ml log phase cells was spread on a Sauton's agar plate, and 200 μg 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 mc^{2}7000 was grown to an OD of 0.5. Cells were harvested, washed with PBS, and diluted to 10^8 cfu/ml in PBST. 8 μM TDMH was added to 1 ml of cell suspension and rotated at 37 °C. At regular time intervals, 100 μl samples were collected, and ATP content was determined by adding 100 μl of ENLITEN luciferase/luciferin reagent (Promega), and the luminescence was measured using a Monolight 2010 luminometer.

Lipid Analysis—mc^{2}7000 cultures (OD of 0.5) were labeled with [^{14}C]acetate for 6 h, washed, resuspended in 250 μl of PBST at a density of 10^9 cfu/ml, and 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 previously (27). The extracted lipids equivalent to 5000 cpm from each sample were spotted on a one-dimensional TLC and developed in either $CHCl_3$/MeOH (97:3, v/v) for FM analysis, $CHCl_3$/MeOH/H_2O (90:10:1, v/v/v) for TDM analysis, or $CHCl_3$/MeOH/NH_4OH (80:20:2, v/v/v) for TMM analysis, as described earlier (13, 27, 29). Endogenously purified [^{14}C]FM, [^{14}C]TDM, and [^{14}C]TMM were used as standards. For two-dimensional 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, 100 μg of TDM or 60 μg of TMM were sonicated in 100 μl of PBST prior to adding 50 μg of TDMH (corresponding to 20-fold molar excess of lipid to the enzyme) to a final volume of 125 μl. The lipid/enzyme mixture

was incubated for 30 min, prior to adding it to a 125-μl suspension of ^{14}C-labeled *M. tuberculosis* cells at 10^9 cfu/ml.

In Vitro Catalytic Activity of TDMH—[^{14}C]TDM or [^{14}C]TMM, equivalent to 100,000 cpm, was homogeneously suspended in the assay buffer as described earlier (27). The homogenate was then mixed with either 5 μg of TDMH, 5 μg of TDMH (S124A), or an equal volume of storage buffer and incubated at 37 °C for 2 h. 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 $CHCl_3$/MeOH (97:3, v/v) (27).

Nucleic Acid Detection and RT-PCR—For determining nucleic acids, 10 μl of 10^7 cfu/ml *M. tuberculosis* (Erdman) was mixed with either 1 μl of 13 μg TDMH or 1 μl of storage buffer in PBST supplemented with 26 mM L-asparagine (PBSTA) and incubated at 37 °C. At various time intervals, the reactions were heat-inactivated at 80 °C for 20 min and centrifuged at 14,000 rpm for 30 s, and 1 μl of the reaction was placed in a nanophotometer to measure the NA contents. For RT-PCR, 10 μl of 10^4 cfu/ml *M. tuberculosis* (Erdman) were mixed with either 13 μg of TDMH or an equivalent volume of storage buffer in PBSTA, incubated for 30 min at 37 °C, and then heat-inactivated at 80 °C for 20 min. After centrifugation at 14,000 rpm for 30 s, 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 500 nM each of the *M. tuberculosis* 16 S rRNA-specific forward and reverse primers, 200 nM molecular beacon, and 5 μl of 2× RT-PCR master mix (Applied Biosystems). The amplification conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 60 s, and 72 °C for 30 s in a StepOnePlus RT-PCR System (Applied Biosystems). The forward and reverse primer sequences were 5′-GAGATACTCGAGTGGCGAAC-3′ and 5′-GGCCGGCTACCCGTCGTC-3′, respectively, and the molecular beacon consisted of a 5′-fluorescein-GCGCCCGC-GGCCTATCAGCTTGTTGGTGGCGC-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—mc^{2}7000 cells (10^9 cfu/ml) were mixed with 8 μM TDMH in PBST, harvested after a 12-h incubation, put on ice, and transferred to Leica EMPACT 2 (Leica Microsystems) membrane carriers (1.5 × 0.2 mm) for high pressure freezing in EMPACT 2 (Leica), at an average pressure of 2000 bars (31). The frozen cells were then transferred to a freeze-substitution machine, Leica EM AFS (Leica), for a 5-day solvent substitution. Briefly, frozen samples were warmed up from −196 to −90 °C over 3 days in a precooled (−90 °C) 1% OsO_4 and 0.1% uranyl acetate mixture dissolved in acetone. Samples were then gradually warmed up to room temperature over 18 h 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 min, followed by Reynold's lead citrate (32) for 7 min, and were examined with a Tecnai F20 electron microscope (FEI) equipped with a 4k × 4k camera (Gatan).

RESULTS

Exposure to TDMH Leads to a Rapid Loss of M. tuberculosis Viability—Approximately 10^6 cfu/ml *M. tuberculosis* H37Rv were incubated with increasing concentrations of the purified recombinant TDMH for 24 h in PBST. Fig. 1*A* shows that viability of the population was reduced by at least 100-fold after exposure to 0.8 μM or higher concentrations of the enzyme (Fig. 1*A*). At a 10-fold excess concentration (8 μM), the enzyme could reduce the viability of 10^8 cfu/ml of bacilli in less than 2 h, but higher density (10^9 cfu/ml) required longer periods of exposure (Fig. 1*B*).

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. 1*C*), although no protein degradation was observed under these conditions (data not shown). Removal of the albumin-based supplement (OADC) restored TDMH activity in both the 7H9 and DMEM base but not to the levels observed in Sauton's broth medium (Fig. 1*C*). 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 envelopes (34). We therefore reasoned that a high concentration (26 mM) 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. 1*D*). To exclude the possible interference of Tween 80, known to influence the properties of mycobacterial envelope (35), we tested the activity of TDMH in PBS without Tween 80 and found no significant change in lytic activity of the enzyme (Fig. 1*E*). 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 3 weeks of incubation at 37 °C (Fig. 1*F*). 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 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 8 μM TDMH for 48 h (Fig. 2*A*). Lysis was further confirmed by the release of ATP from TDMH-exposed *M. tuberculosis* mc^{2}7000 (Fig. 2*B*). The timing of ATP release in Fig. 2*B* was also consistent with the loss of cell viability at this density (Fig. 1*B*). We examined the integrity of TDMH-treated bacteria at an ultrastructural level by high pressure freezing and freeze-substitution electron microscopy. As expected, a distinct multiple layered architec-

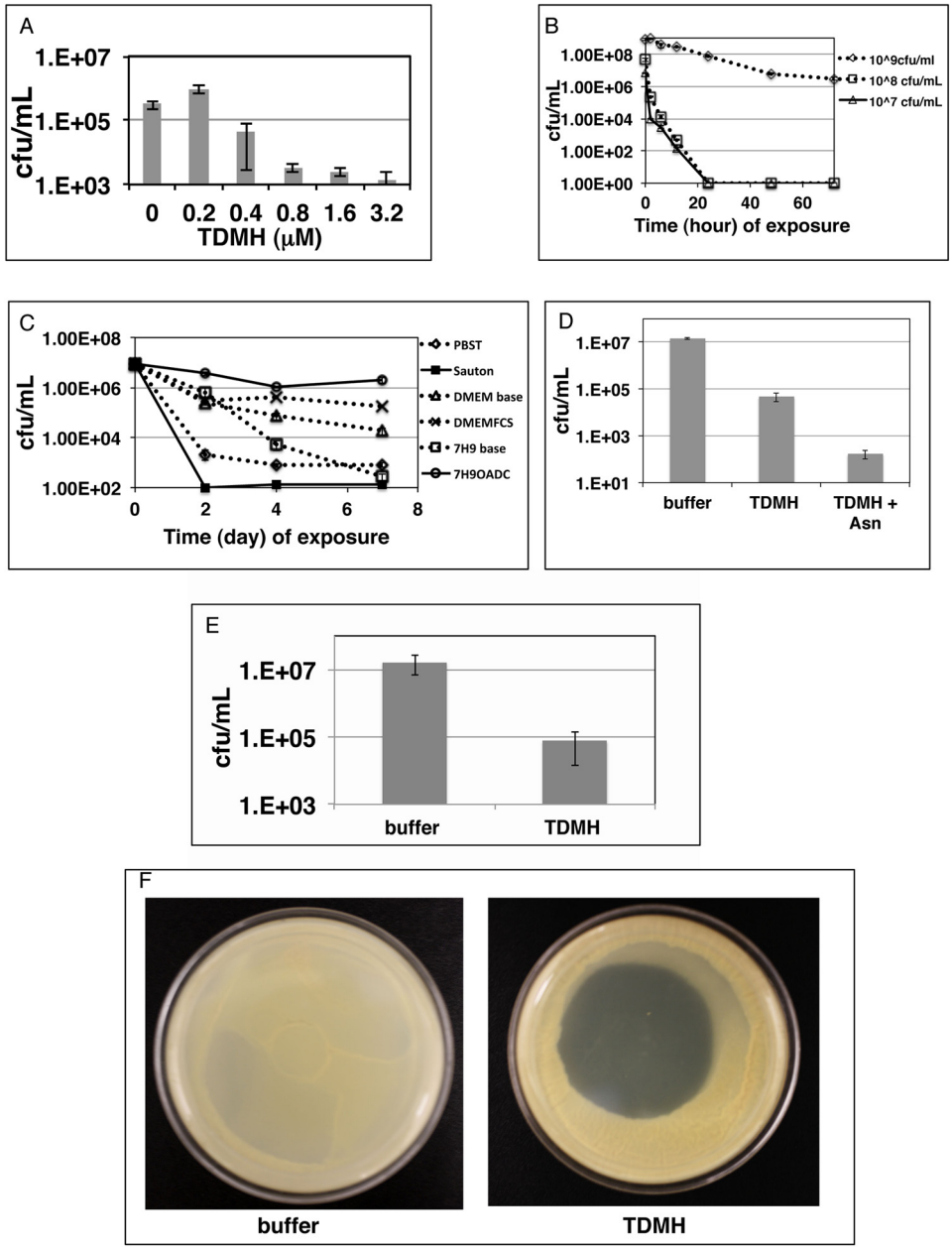


FIGURE 1. **Loss of *M. tuberculosis* viability upon TDMH exposure.** *A,* viability of 10^6 cfu/ml bacilli after exposure to various concentrations of TDMH for 24 h in PBST. In the control (0 μM) experiment, an equivalent volume of storage buffer was used. *B,* exposure of various densities of *M. tuberculosis* ($mc^2$7000) to 8 μM TDMH over 72-h 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 L-Asn) on the antimycobacterial activity of TDMH in PBST. *E,* lytic activity of TDMH against *M. tuberculosis* in detergent-free PBS after a 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.

ture of the envelope, including an outer layer, was observed in the untreated bacteria (Fig. 2*C*, *panels i–iii*). In the TDMH-exposed bacilli, the outer layer was visibly ablated at several places (Fig. 2*C*, *panels iv–viii*). We could also capture cells that were either in the process of losing the cytoplasmic content during lysis (Fig. 2*C*, *panels v–vii*) or had completely lost the cytosolic content and the outer cell envelope (Fig. 2*C*, *panel viii*). The heterogeneous state of TDMH-treated bacilli and the partial loss of the envelope integrity can be attributed to the high density (10^9 cfu/ml) of bacilli exposed to subsaturating concentrations (8 μM) of the enzyme as indicated by the slower lysis (Fig. 1*B*). These observations together are fully consistent with the idea that exposure to TDMH breaches the integrity of the cell envelope outer layer, thereby triggering bacterial lysis. Henceforth, activity of each TDMH preparation was determined by turbidometric 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 nonresponsive 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. 2*D*).

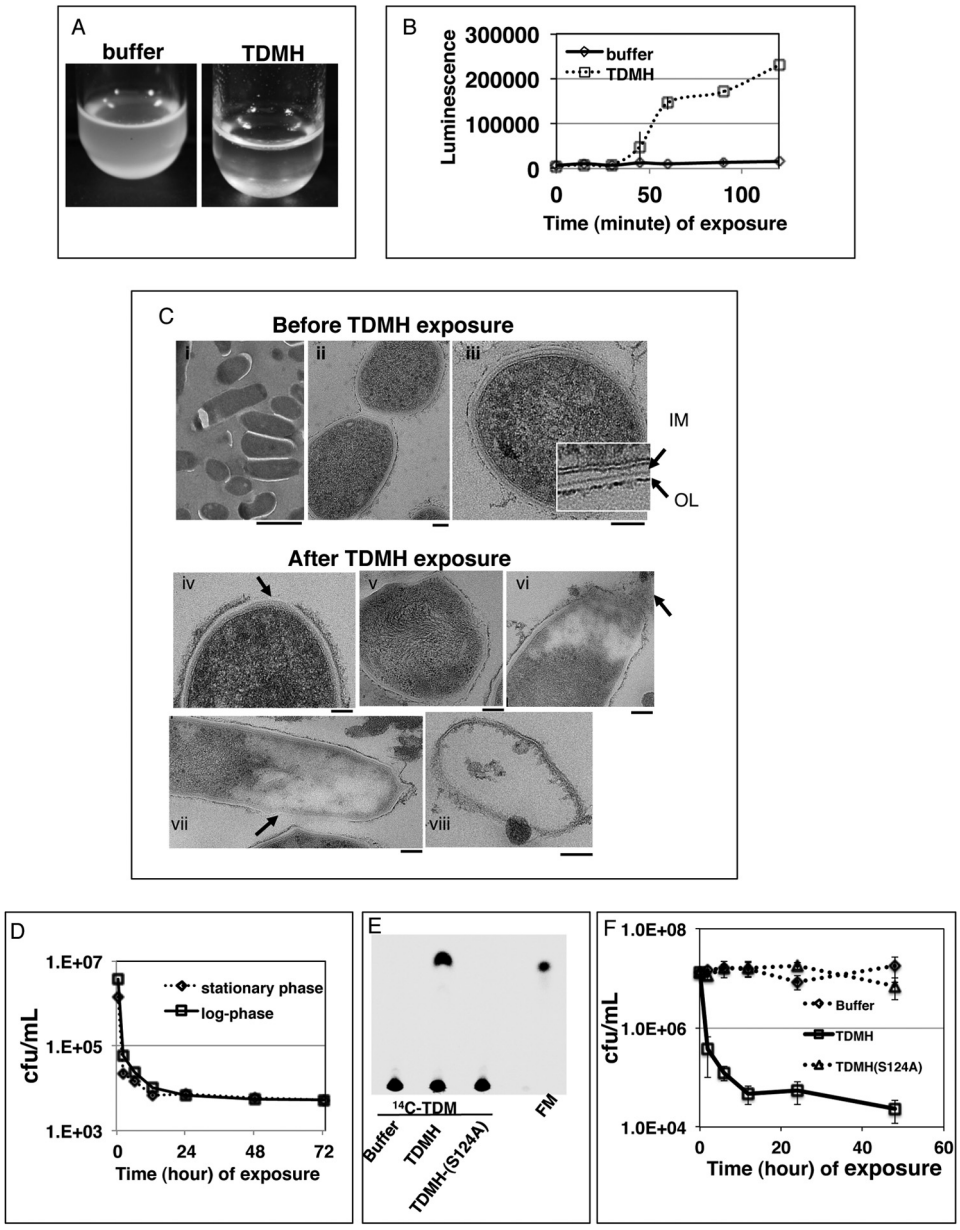


FIGURE 2. **TDMH exposure causes lysis of *M. tuberculosis*.** *A,* clearance of *M. tuberculosis* (H37Rv) suspension (10^8 cfu/ml) after 48 h in PBST containing 8 μM TDMH. In the control, an equal volume of storage buffer was added in the cell suspension. *B,* luciferin-luciferase-based measurement of ATP released in an *M. tuberculosis* ($mc^2$7000) suspension (10^8 cfu/ml) exposed to 8 μM TDMH (also see Fig. 1*B*). As a negative control, ATP was measured in culture exposed to storage buffer. *C,* high pressure freezing and freeze-substitution electron microscopy of 10^9 cfu/ml *M. tuberculosis* ($mc^2$7000) before (*panels i–iii*) and after a 12-h exposure to 8 μM TDMH (*panels iv–viii*). Images in *panels i–iii* are independent fields showing low magnification of intact cells (*panel 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 *panel iii*. The cell inner membrane and outer layer are marked as *IM* and *OL*. Images in *panels iv–viii* are independent fields showing various stages of lysing bacilli in a TDMH-exposed population. Bacteria shown are as follows: *panel iv*, with a lesion in the outer layer (marked with *arrowhead*); *panel v,* with a distorted cytoplasm, presumably moments before the cytoplasm ejection due to the membrane lesion; *panels vi* and *vii,* in the act of releasing cytoplasmic contents, and *panel viii,* completely devoid of cytoplasm. *Arrows* point to the lesion sites. *Scale bars* are 100 nm for *panels ii–viii*, and 1 μm for *panel 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 8 μM 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 8 μM TDMH (S124A). The *error bars* represent the standard errors of three independent experiments.

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 G*X*S*X*G motif), aspartate, and histidine (27). A catalytically inactive TDMH mutant was generated by replacing the catalytic Ser-121 by an Ala residue. As shown in Fig. 2*E*, TDMH(S124A) clearly failed to hydrolyze TDM (Fig. 2*E*). Importantly, exposure of *M. tuberculosis* to the mutant enzyme failed to lyse the culture (Fig. 2*F*). It can therefore be inferred that exposure to the TDM hydrolase activity caused envelope rupture and mycobacterial lysis.

Depletion of TMM and TDM during Early Phase of TDMH Exposure—Because TDMH hydrolyzes TDM *in vitro* to release FM (27), we next analyzed the levels of FM and TDM in enzyme-exposed *M. tuberculosis* cultures. To this aim, a high

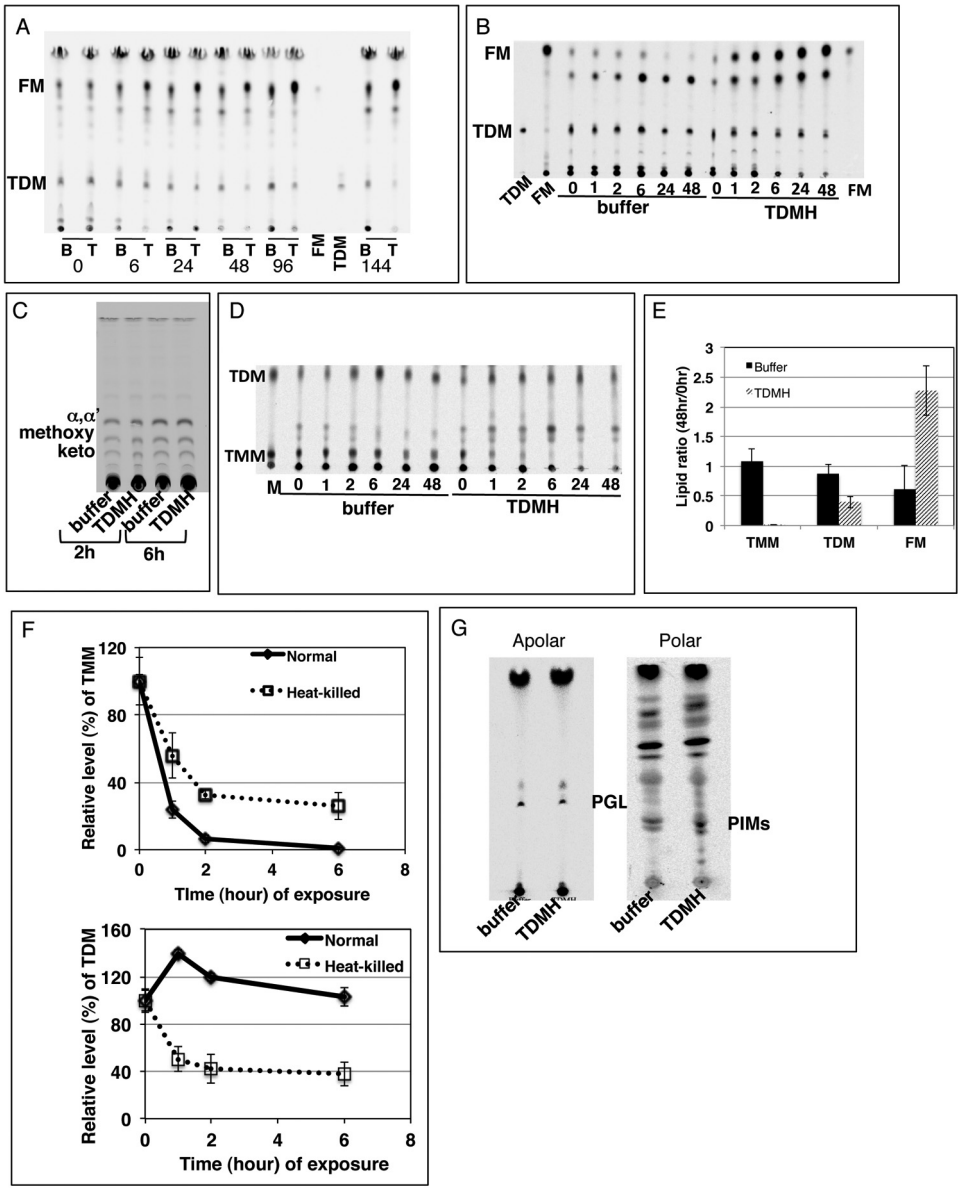


FIGURE 3. **TDMH exposure triggers early release of FM and concomitant depletion of TMM.** *A,* radio-TLC of the lipids in cell/TDMH mixture (marked as *T*) incubated for specified hours (marked *below* each *lane*). Purified [^{14}C]FM and [^{14}C]TDM were loaded as markers. In the parallel control 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 *below* each *lane*). Purified [^{14}C]TDM and [^{14}C]FM were loaded as markers. TLCs in *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 2 and 6 h. *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 a 48-h exposure in buffer and TDMH-exposed *M. tuberculosis*. The ratio between 0 (t_0) and a 48-h exposure was 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 6 h of exposure. The value at each time point, obtained from densitometric analysis of TLCs, represents the percentage of the lipid relative to t_0 (zero hour). The *error bars* denote standard error from two and three independent experiments for normal and heat-treated cells, respectively. *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 2 h. The major apolar and polar glycolipids, phenolic glycolipids (*PGL*), and PIMs are marked. For experiments in *A*–*G*, 10^9 cfu/ml *M. tuberculosis* were exposed to 8 μM TDMH.

bacterial density (10^9 cfu/ml) was used to obtain sufficient lipids in a small volume with an effective amount (8 μM) of TDMH. Because lysis is slowed at this high cell density (Fig. 1*B*), the reactions were followed for an extended period of 6 days. The relative levels of FM in the TDMH/bacteria mixture progressively increased, although it remained almost unchanged in buffer-treated cells (Fig. 3, *A* and *B*). After a 48-h exposure, the FM levels were over twice the amounts present at the beginning of the exposure (Fig. 3*E*). Interestingly, although the level of FM started to accumulate very early, within the first hour of treatment, the depletion of TDM was essentially apparent only after a 48-h exposure, when its level reduced over 50% to the amount seen at the beginning (Fig. 3, *A*, *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 cells to replenish TDM. Besides TDM,

mAGP and TMM are the two most dominant esters of mycolic acids in the mycobacterial cell wall. No significant change in the mycolyl content of mAGP was observed in TDMH-exposed cells during FM release (Fig. 3*C*), consistent with the lack of *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. Fig. 3*D* shows that the levels of TMM in TDMH-exposed cells indeed started to progressively decrease within 1 h of exposure, concomitant with the release of FM seen in Fig. 3*B*.

Because no loss of viability was observed during the first 2 h of exposure under these conditions (Fig. 1*B*), TMM depletion and FM release are very likely to be the pre-lysis events, suggesting that noncovalently 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 ^{14}C-labeled *M. tuberculosis* treated with either buffer or 8 μM TDMH for 2 h. 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. 4*A*). However, we noted elevated levels of phosphatidylinositol and an unknown polar lipid in TDMH-exposed bacilli (Fig. 4*A*). 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 2 h of TDMH exposure. It is, however, noteworthy that in contrast to TDM, purified TMM could not be hydrolyzed by TDMH *in vitro* (Fig. 4*B*). This is rather surprising and difficult to reconcile with the *in vivo* effect of TDHM on cellular TMM. One possibility could be that TDM 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 hour when heat-inactivated cells were exposed to the enzyme (Fig. 3*F*). In addition, we also observed a slower and less pronounced depletion of TMM in the TDMH-exposed heat-killed bacilli (Fig. 3*F*). However, depletion of TMM remained significant in heat-killed cells, a decrease by about 40% within the first hour. No other glycolipids were altered in the enzyme-exposed heat-killed cells during the first 2 h of exposure (Fig. 3*G*).

To further substantiate the impact of the TDMH on cellular TDM, we next analyzed the effect of the exogenous addition of purified TDM on the enzyme-exposed *M. tuberculosis*. Addition of a 20-fold molar excess of purified TDM with respect to TDMH reduced the release of FM from the bacilli by about 30% within 2 h of exposure (Fig. 5*A*). However, a comparable molar excess of exogenous TMM had no effect on FM release (Fig. 5*A*), consistent with the inability of the enzyme to hydrolyze purified TMM *in vitro* (Fig. 4*B*). Similarly, a 200-fold molar excess of TDM with respect to TDMH could abrogate the lytic activity of the enzyme (Fig. 5*B*). The inhibition in the presence of equivalent weight of a TDM-containing apolar lipid fraction

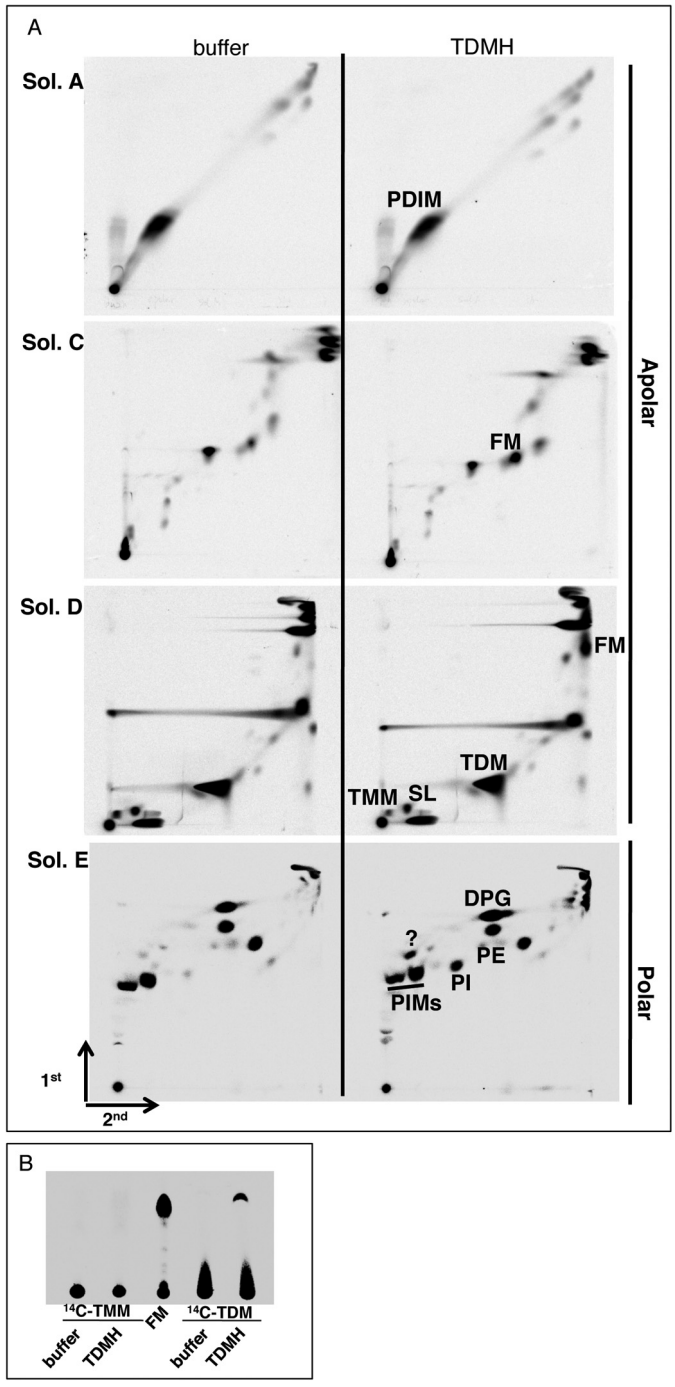


FIGURE 4. *A*, two-dimensional radio-TLC of total apolar and polar lipids equivalent to 35,000 cpm extracted from cells treated with either buffer or TDMH for 2 h. Apolar lipids were resolved in solvent (*Sol.*) systems A, C, and D, and polar lipids were resolved in solvent system E as described previously (39). Positions of phthiocerol dimycocerosic acid (*PDIM*), PIMs, phosphatidylinositol (*PI*), phosphatidylethanolamine (*PE*), and diphosphatidylglycerol (*DPG*) are marked based on Ref. 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 [^{14}C]TDM, but not [^{14}C]TMM, is incubated with 5 μg of TDMH.

was partial (Fig. 5*B*). However, neither polar lipid nor purified mAGP and PIM, a major polar lipid of the cell envelope (Fig. 4*A*), had any effect on the lysis (Fig. 5*B*). The failure of mAGP to inhibit the lytic activity is consistent with the following: (*a*) the lack of any apparent loss of its mycolyl content in the exposed

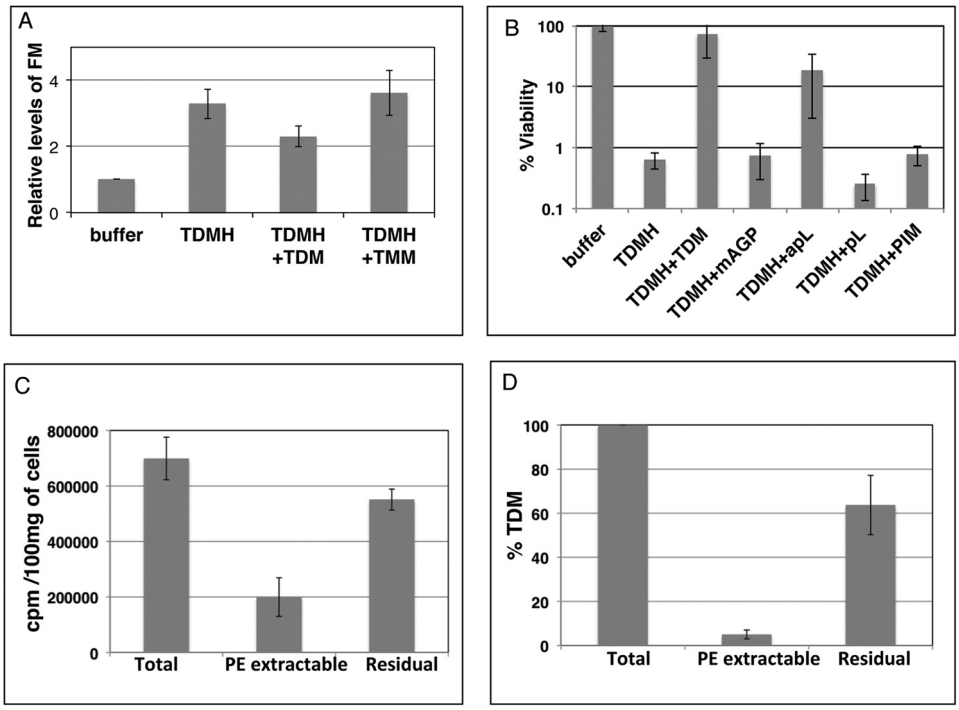


FIGURE 5. **TDM is an early target of TDMH.** *A,* quantitative analysis of relative change in FM levels in *M. tuberculosis*/TDMH mixture with respect to the buffer-exposed control, as described in *A*. *Error bar* indicates standard error of the densitometric data obtained from three independent experiments. *B,* lytic activity of 0.8 μM 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 of viability in each condition was calculated using buffer-treated cells as reference (100%). *C,* total radio lipids (counts/min) extracted from 100 mg of ^{14}C-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.

cell (Fig. 3*C*), 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. The requirement of a large molar excess of purified TDM for an apparent inhibition of 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 in its 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 the following: (*a*) petroleum ether extracts; (*b*) the residual lipids obtained by re-extraction of petroleum ether treated cells in $CHCl_3$/MeOH (2:1, v/v), and (*c*) total lipid extracts from cells in $CHCl_3$/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. 5*C*). 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 glycolipids were re-extracted along with the residual lipids of the ether-treated bacteria (Fig. 5*D*), 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 as to 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 the *M. tuberculosis* complex as well as nontuberculous mycobacteria. 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. 6*A*). Because mycolyl esters of trehalose are unique lipids of mycobacteria, TDMH exposure, as expected, had no effect on *E. coli* (Fig. 6*A*). 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, it 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 in the presence of shorter chain α' and absence of methoxy and keto species (11). Interestingly, *M. marinum* appears very sensitive to TDMH (Fig. 6*A*). The failure of TDMH(S124A) to lyse *M. marinum* cells (Fig. 6*B*) suggests that depletion of trehalose mycolates could be the likely trigger of lysis in this species as well.

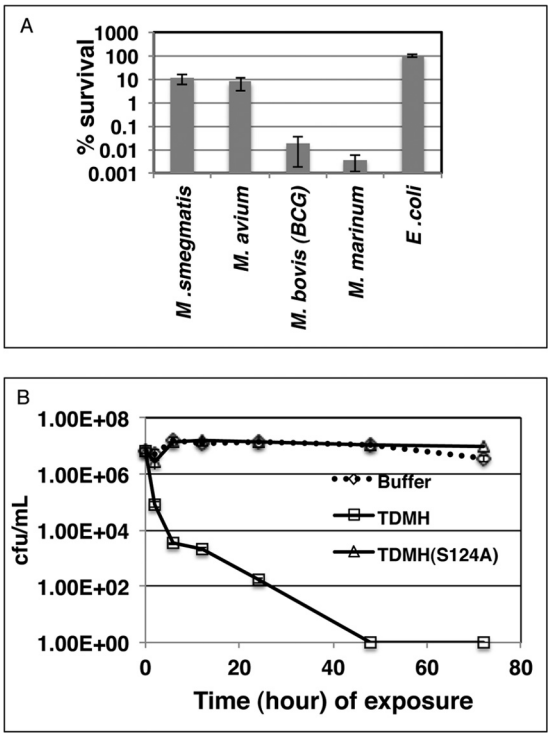


FIGURE 6. **Sensitivity of various mycobacterial species to TDMH.** *A,* lytic activity of 8 μM TDMH against 10^7 cfu/ml of four other mycobacterial species and *E. coli*. The percentage survival reflects the ratio of the number of viable bacilli before and after the 48-h exposure. *B,* effect on the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

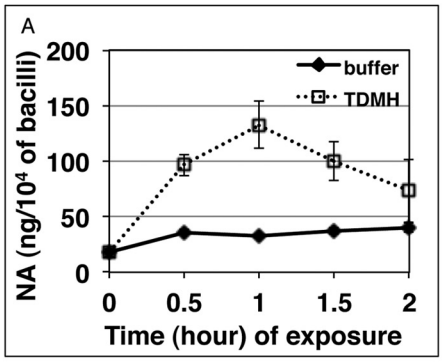
TDMH Facilitates M. tuberculosis Detection at Low Density—A rapid and efficient lysis of TDMH exposed *M. tuberculosis,* and a subsequent release of nucleic acids (NA) offers a unique opportunity to use this enzyme for achieving more sensitive detection in a nucleic acid amplification assay for TB diagnosis. NA content in the supernatant of TDMH-exposed bacilli indeed started to increase within 30 min of exposure, and it peaked at around 60 min (Fig. 7*A*). 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 the spontaneous lysis of bacteria at low frequency during heating (Fig. 7*A*). We next performed a molecular beacon-based 40-cycle real time-PCR (30) to detect NA corresponding to 16 S 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 (Fig. 7, *B* and *C*). However, treatment of samples with TDMH prior to the amplification reaction facilitated the NA detection in 37 samples, a highly significant ($p < 0.0001$) improvement in the numbers of positive detection (Fig. 7*C*). Low frequency of NA detection in some of the untreated samples is consistent with the Poisson distribution pattern of dilution in which some dilutions have large enough bacilli to generate sufficient amounts of template due to autolysis during heating.

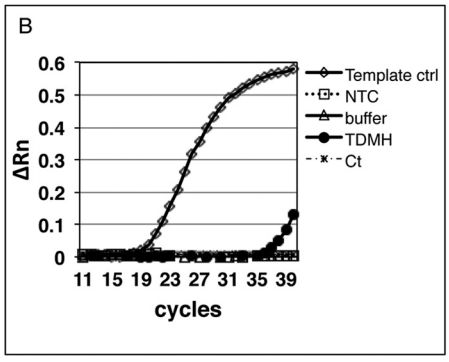
DISCUSSION

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 indicates that these lipids are both exposed on the 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 (~8 nm) of the mycomembrane or in a 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 the 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 TDMH 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 toward 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





C

p<0.0001

Dilutions	buffer			TDMH		
	R1	R2	R3	R1	R2	R3
D1	–	–	37	–	38	35
D2	–	–	–	35	37	36
D3	–	–	37	–	36	37
D4	–	38	37	37	37	37
D5	–	–	–	37	37	37
D6	–	–	–	38	38	38
D7	–	–	–	37	36	38
D8	–	35	–	36	36	37
D9	–	–	–	37	37	36
D10	39	–	–	37	36	38
D11	–	–	–	36	–	38
D12	–	–	37	34	37	–
D13	–	–	–	38	–	–
D14	–	–	–	37	37	37
D15	–	–	–	–	–	37

FIGURE 7. **TDMH treatment of *M. tuberculosis* releases nucleic acids and facilitates sensitive detection by RT-PCR.** *A,* 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* NA corresponding to 16 S rRNA in molecular beacon-based RT-PCR performed on samples with ~10 bacilli either treated with TDMH or storage buffer control. Controls with genomic DNA of *M. tuberculosis* as template (*template ctrl*) or without any template (*NTC*) contain equivalent amounts of TDMH. *C,* summary table of threshold cycles where the signal was detected in 45 independent RT-PCRs 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.

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.

Acknowledgments—We thankfully acknowledge the gifts of M. marinum from Pamela Small and *M. avium* from Delphi Chatterjee, statistical analysis of the results by Abdus Wahed, and critical comments on the manuscript by Graham Hatfull. We also acknowledge excellent technical support from Kathleen Kulka.

REFERENCES

1. Grange, J. (1996) *Mycobacteria and Human Disease*, 2nd Ed., pp. 41–55, Oxford University Press, New York
2. Dye, C., Lönnroth, K., Jaramillo, E., Williams, B. G., and Raviglione, M. (2009) Trends in tuberculosis incidence and their determinants in 134 countries. *Bull. World Health Organ.* **87,** 683–691
3. Shinnick, T. M., and Good, R. C. (1994) Mycobacterial taxonomy. *Eur. J. Clin. Microbiol. Infect. Dis.* **13,** 884–901
4. Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., Edelstein, P. H., Cosma, C. L., and Ramakrishnan, L. (2011) Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* **145,** 39–53
5. Ehrt, S., and Schnappinger, D. (2009) Mycobacterial survival strategies in the phagosome. Defence against host stresses. *Cell. Microbiol.* **11,** 1170–1178
6. Brennan, P. J., and Nikaido, H. (1995) The envelope of mycobacteria. *Annu. Rev. Biochem.* **64,** 29–63
7. Nikaido, H., and Jarlier, V. (1991) Permeability of the mycobacterial cell wall. *Res. Microbiol.* **142,** 437–443
8. Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C., and Engelhardt, H. (2010) Mycobacterial outer membranes: in search of proteins. *Trends Microbiol.* **18,** 109–116
9. Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., and Engelhardt, H. (2008) Disclosure of the mycobacterial outer membrane. Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 3963–3967
10. Ortalo-Magné, A., Lemassu, A., Lanéelle, M. A., Bardou, F., Silve, G., Gounon, P., Marchal, G., and Daffé, M. (1996) Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species. *J. Bacteriol.* **178,** 456–461
11. Takayama, K., Wang, C., and Besra, G. S. (2005) Pathway to synthesis and

processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* **18,** 81–101
12. Grzegorzewicz, A. E., Pham, H., Gundi, V. A., Scherman, M. S., North, E. J., Hess, T., Jones, V., Gruppo, V., Born, S. E., Korduláková, J., Chavadi, S. S., Morisseau, C., Lenaerts, A. J., Lee, R. E., McNeil, M. R., and Jackson, M. (2012) Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane. *Nat. Chem. Biol.* **8,** 334–341
13. Belisle, J. T., Vissa, V. D., Sievert, T., Takayama, K., Brennan, P. J., and Besra, G. S. (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* **276,** 1420–1422
14. Puech, V., Guilhot, C., Perez, E., Tropis, M., Armitige, L. Y., Gicquel, B., and Daffé, M. (2002) Evidence for a partial redundancy of the fibronectin-binding proteins for the transfer of mycoloyl residues onto the cell wall arabinogalactan termini of *Mycobacterium tuberculosis*. *Mol. Microbiol.* **44,** 1109–1122
15. Jackson, M., Raynaud, C., Lanéelle, M. A., Guilhot, C., Laurent-Winter, C., Ensergueix, D., Gicquel, B., and Daffé, M. (1999) Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol. Microbiol.* **31,** 1573–1587
16. Hattori, Y., Matsunaga, I., Komori, T., Urakawa, T., Nakamura, T., Fujiwara, N., Hiromatsu, K., Harashima, H., and Sugita, M. (2011) Glycerol monomycolate, a latent tuberculosis-associated mycobacterial lipid, induces eosinophilic hypersensitivity responses in guinea pigs. *Biochem. Biophys. Res. Commun.* **409,** 304–307
17. Matsunaga, I., Naka, T., Talekar, R. S., McConnell, M. J., Katoh, K., Nakao, H., Otsuka, A., Behar, S. M., Yano, I., Moody, D. B., and Sugita, M. (2008) Mycolyltransferase-mediated glycolipid exchange in *Mycobacteria*. *J. Biol. Chem.* **283,** 28835–28841
18. Rombouts, Y., Brust, B., Ojha, A. K., Maes, E., Coddeville, B., Elass-Rochard, E., Kremer, L., and Guerardel, Y. (2012) Exposure of mycobacteria to cell wall-inhibitory drugs decreases production of arabinoglycerolipid related to mycolyl-arabinogalactan-peptidoglycan metabolism. *J. Biol. Chem.* **287,** 11060–11069
19. Tahlan, K., Wilson, R., Kastrinsky, D. B., Arora, K., Nair, V., Fischer, E., Barnes, S. W., Walker, J. R., Alland, D., Barry, C. E., 3rd, and Boshoff, H. I. (2012) SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **56,** 1797–1809
20. Noll, H., Bloch, H., Asselineau, J., and Lederer, E. (1956) The chemical structure of the cord factor of *Mycobacterium tuberculosis*. *Biochim. Biophys. Acta* **20,** 299–309
21. Warrier, T., Tropis, M., Werngren, J., Diehl, A., Gengenbacher, M., Schlegel, B., Schade, M., Oschkinat, H., Daffe, M., Hoffner, S., Eddine, A. N., and Kaufmann, S. H. (2012) Antigen 85C inhibition restricts *Mycobacterium tuberculosis* growth through disruption of cord factor biosynthesis. *Antimicrob. Agents Chemother.* **56,** 1735–1743
22. Harth, G., Zamecnik, P. C., Tabatadze, D., Pierson, K., and Horwitz, M. A. (2007) Hairpin extensions enhance the efficacy of mycolyl transferase-specific antisense oligonucleotides targeting *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 7199–7204
23. Hunter, R. L., Olsen, M. R., Jagannath, C., and Actor, J. K. (2006) Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavitary tuberculosis, including a revised description of the pathology of secondary disease. *Ann. Clin. Lab. Sci.* **36,** 371–386
24. Geisel, R. E., Sakamoto, K., Russell, D. G., and Rhoades, E. R. (2005) *In vivo* activity of released cell wall lipids of *Mycobacterium bovis* bacillus Calmette-Guérin is due principally to trehalose mycolates. *J. Immunol.* **174,** 5007–5015
25. Fujita, Y., Naka, T., McNeil, M. R., and Yano, I. (2005) Intact molecular characterization of cord factor (trehalose 6,6′-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* **151,** 3403–3416
26. Kai, M., Fujita, Y., Maeda, Y., Nakata, N., Izumi, S., Yano, I., and Makino, M. (2007) Identification of trehalose dimycolate (cord factor) in *Mycobacterium leprae*. *FEBS Lett.* **581,** 3345–3350
27. Ojha, A. K., Trivelli, X., Guerardel, Y., Kremer, L., and Hatfull, G. F. (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *J. Biol. Chem.* **285,** 17380–17389
28. Nelson, D., Loomis, L., and Fischetti, V. A. (2001) Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4107–4112
29. Besra, G. S. (1998) in *Mycobacteria Protocols* (Parish, T., and Stoker, N. G., eds) pp. 91–107, Humana Press Inc., Totowa, NJ
30. El-Hajj, H. H., Marras, S. A., Tyagi, S., Kramer, F. R., and Alland, D. (2001) Detection of rifampin resistance in *Mycobacterium tuberculosis* in a single tube with molecular beacons. *J. Clin. Microbiol.* **39,** 4131–4137
31. Studer, D., Graber, W., Al-Amoudi, A., and Eggli, P. (2001) A new approach for cryofixation by high pressure freezing. *J. Microsc.* **203,** 285–294
32. Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208–212
33. Parish, T., and Wheeler, P. (1998) in *Mycobacteria Protocols* (Parish, T., and Stoker, N., eds) pp. 77–90, Humana Press Inc., Totowa, NJ
34. Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010) D-amino acids trigger biofilm disassembly. *Science* **328,** 627–629
35. Van Boxtel, R. M., Lambrecht, R. S., and Collins, M. T. (1990) Effects of colonial morphology and Tween 80 on antimicrobial susceptibility of *Mycobacterium paratuberculosis*. *Antimicrob. Agents Chemother.* **34,** 2300–2303
36. Bloch, H. (1950) Studies on the virulence of tubercle bacilli; isolation and biological properties of a constituent of virulent organisms. *J. Exp. Med.* **91,** 197–218
37. Villeneuve, M., Kawai, M., Kanashima, H., Watanabe, M., Minnikin, D. E., and Nakahara, H. (2005) Temperature dependence of the Langmuir monolayer packing of mycolic acids from *Mycobacterium tuberculosis*. *Biochim. Biophys. Acta* **1715,** 71–80
38. Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffé, M., and Guilhot, C. (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 314–319
39. Ojha, A. K., Baughn, A. D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jacobs, W. R., Jr., and Hatfull, G. F. (2008) Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol.* **69,** 164–174
40. Brust, B., Lecoufle, M., Tuaillon, E., Dedieu, L., Canaan, S., Valverde, V., and Kremer, L. (2011) *Mycobacterium tuberculosis* lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis. *PLoS ONE* **6,** e25078