Photoactivatable glycolipid probes for identifying mycolate–protein interactions in live mycobacteria

Herbert W. Kavunja,[1] Kyle J. Biegas,[1] Nicholas Banahene,[1] Jessica A. Stewart,[1] Brent F. Piligian,[1] Jessica M. Groenevelt,[1] Caralyn E. Sein,[2] Yasu S. Morita,[2,3] Michael Niederweis,[4] M. Sloan Siegrist,[2,3] and Benjamin M. Swarts[1,*]

[1]Department of Chemistry and Biochemistry, Central Michigan University, Mount Pleasant, MI, USA

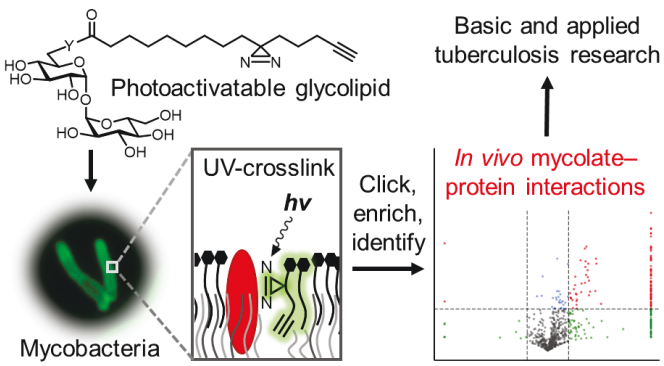
[2]Department of Microbiology, University of Massachusetts, Amherst, MA, USA

[3]Molecular and Cellular Biology Graduate Program, University of Massachusetts, Amherst, MA, USA

[4]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

*Corresponding author: E-mail: ben.swarts@cmich.edu

TOC Graphic



Word count (maximum 2,200): 2,198

Abstract

Mycobacteria have a distinctive glycolipid-rich outer membrane, the mycomembrane, which is a critical target for tuberculosis drug development. However, proteins that associate with the mycomembrane, or that are involved in its metabolism and host interactions, are not well-characterized. To facilitate the study of mycomembrane-related proteins, we developed photoactivatable trehalose monomycolate analogues that metabolically incorporate into the mycomembrane in live mycobacteria, enabling *in vivo* photo-crosslinking and click chemistry-mediated analysis of mycolate-interacting proteins. When deployed in *Mycobacterium smegmatis* with quantitative proteomics, this strategy enriched over 100 proteins, including the mycomembrane porin (MspA), several proteins with known mycomembrane synthesis or remodeling functions (CmrA, MmpL3, Ag85, Tdmh), and numerous candidate mycolate-interacting proteins. Our approach is highly versatile, as it: (i) enlists click chemistry for flexible protein functionalization; (ii) in principle can be applied to any mycobacterial species to identify endogenous bacterial proteins or host proteins that interact with mycolates; (iii) can potentially be expanded to investigate protein interactions with other mycobacterial lipids. This tool is expected to help elucidate fundamental physiological and pathological processes related to the mycomembrane and may reveal novel diagnostic and therapeutic targets.

Manuscript Text and Figures

Mycobacteria are of enormous medical and biotechnological importance. The most prominent example is tuberculosis-causing *Mycobacterium tuberculosis* (*Mtb*), which kills 1.5 million people annually and exists in drug-resistant forms that are extremely challenging to treat.[1–4] Underlying the success of *Mtb* and related pathogens is a complex cell envelope containing plasma membrane, peptidoglycan, arabinogalactan, and an outer membrane called the mycomembrane (Figure 1).[5–8] The mycomembrane consists of long, branched mycolic acids, which predominantly exist as mycolate esters linked to carbohydrates.[7–11] The mycomembrane is essential for survival due to its roles in cellular integrity and defense, nutrient acquisition, and cellular communication, including host–pathogen interactions.[9,10] Multiple drugs used to treat tuberculosis act on mycomembrane biosynthesis, highlighting why this membrane is a major focal point for mycobacteria research.[12]

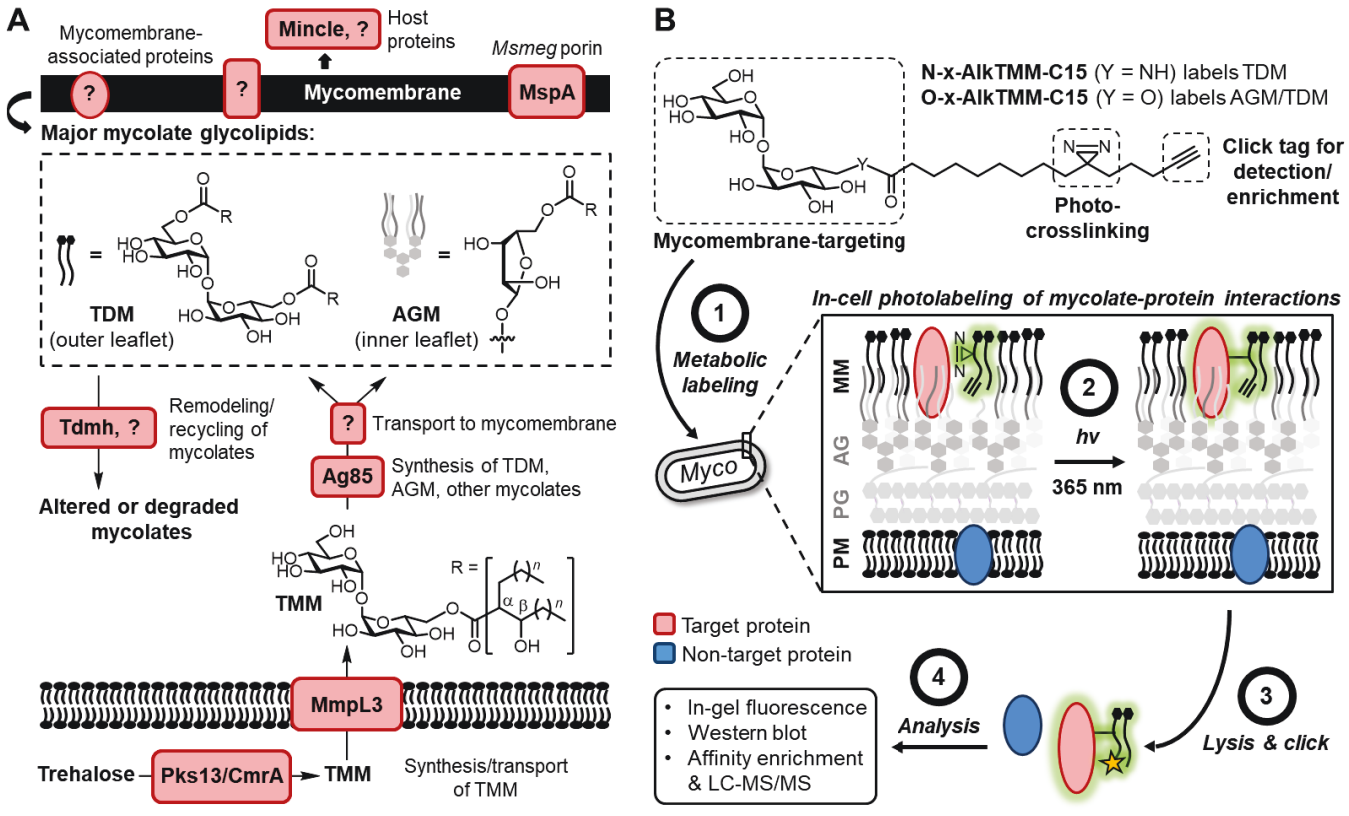


Figure 1. (A) Metabolism and host interactions of mycolate glycolipids. (B) Strategy for *in vivo* capture and analysis of mycolate-interacting proteins using photoactivatable probes (see Scheme S1 and SI Discussion).

Significant progress toward elucidating mycomembrane composition, biosynthesis, and function has been made, although much remains to be learned. Its major mycolate glycolipids, including trehalose monomycolate (TMM), trehalose dimycolate (TDM), and arabinogalactan mycolate (AGM), are synthesized as shown in Figure 1A. TMM is synthesized from trehalose in the cytoplasm via Pks13/CmrA,[13] then exported by MmpL3[14,15] and processed by Ag85 mycoloyltransferases[16–18] to generate TDM and AGM. However, the identities of many proteins involved in mycomembrane lipid transport, remodeling, turnover, and host interactions have remained elusive. Furthermore, the proteomic composition of the mycomembrane is notoriously poorly defined.[19] Despite computational predictions that the *Mtb* genome may encode over 100 mycomembrane-associated proteins,[20–22] only a few have been identified and characterized across the *Mycobacterium* genus.[19,23–27] Most of these proteins exhibit channel activity and/or are important for nutrient influx, including the *M. smegmatis* (*Msmeg*) porin (MspA),[23,28,29] *Mtb* CpnT,[24] and newly discovered *Mtb* PPE51.[30,31] The many as-yet unidentified mycomembrane proteins likely have other critical functions as well, including secretion/efflux processes, cell envelope biosynthesis and remodeling, and host–pathogen interactions.[19]

New tools are needed to accelerate the identification and functional characterization of mycomembrane-related proteins. Significant efforts have been made to enrich and identify mycomembrane-resident proteins,[27,32–36] mainly through subcellular fractionation and detergent extraction, but the resolution of cell envelope layers remains extremely challenging due to the massive peptidoglycan–arabinogalactan–mycolate covalent complex. Moreover, the lysis conditions, detergents, and centrifugations in these methods do not retain all of the protein–lipid interactions that occur *in vivo*, particularly weaker, transient interactions, which are frequently lost.[37] Such methods are also not designed to capture proteins that are not directly associated with the mycomembrane, and thus miss an important subset of proteins involved in mycomembrane metabolism or host interactions. Finally, traditional methods are laborious and often incompatible with complex experimental contexts, e.g. biofilm cultures or macrophage/animal infections. Recently, lipid-mimicking probes bearing photoactivatable and clickable groups have emerged as valuable tools for profiling *in vivo* lipid–protein interactions.[37–41] Here, we merged this photolabeling concept with our mycomembrane-targeting probes to develop the first tool

for global analysis of *in vivo* mycolate–protein interactions, providing a powerful new approach to investigating mycomembrane-related proteins in their native state.

We reported that TMM analogues bearing functionalized mycolate-mimicking chains can metabolically incorporate into mycomembrane components via conserved, substrate-promiscuous Ag85 mycoloyltransferases.[42–44] By altering the linker, we controlled the incorporation mechanism and labeling target, with amide-linked N-AlkTMM-C7 exclusively labeling TDM and ester-linked O-AlkTMM-C7 labeling AGM and TDM (Scheme S1, Supporting Information (SI)).[42] Capitalizing on the TMM scaffold, we designed the two photoactivatable analogues N- and O-x-AlkTMM-C15 to enable mycomembrane proteomics (Figure 1B, SI Discussion). Both analogues possess the mycomembrane-targeting TMM moiety containing a lipophilic chain, which has a photoactivatable diazirine and a clickable alkyne. We envisioned that N- or O-x-AlkTMM-C15 would metabolically embed into glycolipids in live cells, placing the lipophilic chain in proximity to mycomembrane-related proteins. Upon UV photo-activation, the diazirine photo-crosslinks proteins, enabling click-mediated affinity enrichment from cell lysates and subsequent identification. In principle, this strategy enables capture and analysis of proteins that associate directly with the mycomembrane or that are involved in mycolate synthesis, transport, remodeling, turnover, or host interactions (SI Discussion).

The syntheses of both probes employed bifunctional fatty acid **1**,[39] which we conjugated to trehalose derivatives **2** and **3**[45–47] to produce N- and O-x-AlkTMM-C15 in two steps (Figure 2A). Using bovine serum albumin (BSA) as a model protein,[48] we confirmed that both probes possessed the requisite functionalities of (i) photo-crosslinking proteins when UV-irradiated and (ii) labeling and detecting the resulting crosslinked products via Cu-catalyzed azide–alkyne cycloaddition (CuAAC) (Figure 2B).

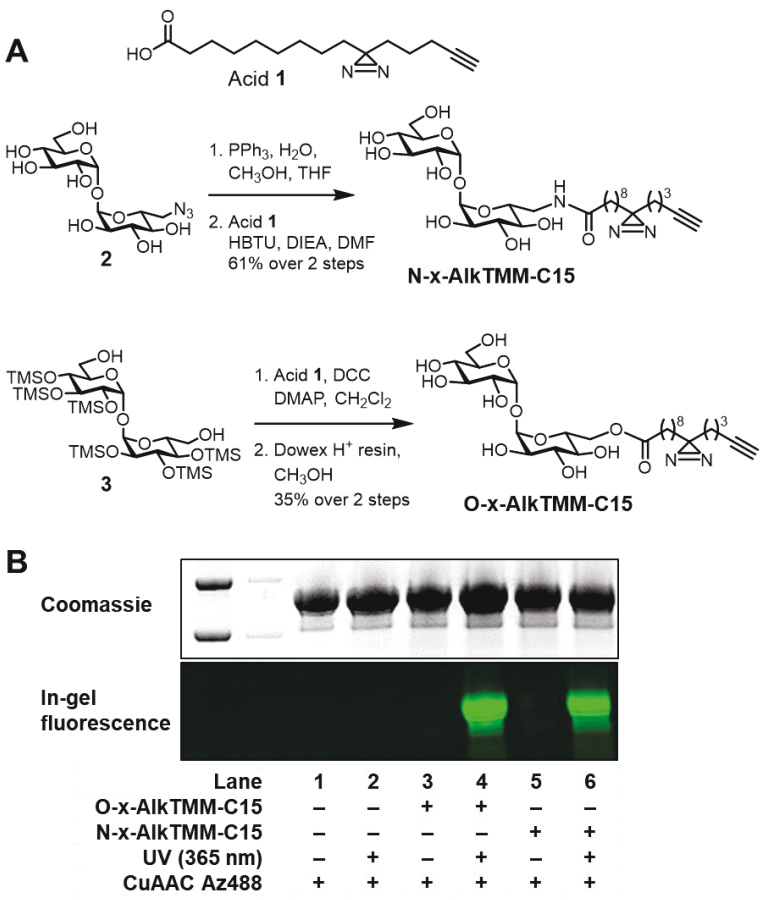


Figure 2. (A) Syntheses of N- and O-x-AlkTMM-C15. (B) UV-dependent photo-crosslinking of BSA with probes followed by CuAAC-mediated product detection.

We tested whether N- and O-x-AlkTMM-C15 metabolically incorporated into the mycomembrane of live bacteria, focusing on the model organism *Msmeg*. Both TMM probes labeled *Msmeg* in a concentration- and time-dependent manner (Figure S1), whereas **1**, which lacks the trehalose targeting moiety, did not label the *Msmeg* surface (Figure S2). Partial growth inhibition for the probes was observed at 250 μM (Figure S3), indicating an optimal concentration of 25–100 μM. The probes were specific, as they efficiently labeled mycomembrane-containing *Msmeg* and *Corynebacterium glutamicum*, but not mycomembrane-deficient *Bacillus subtilis* or *Escherichia coli* (Figures 3A and S4). Consistent with the hypothesized incorporation routes (Scheme S1), N-x-AlkTMM-C15 labeling was entirely localized to the TDM-containing extractable lipids fraction and a new fluorescent lipid consistent with labeled TDM was observed, whereas O-x-AlkTMM-C15 labeling was detected in both the TDM- and AGM-containing fractions (Figures 3B and S5). Signal from both probes, and the peptidoglycan probe RADA[49] (positive

control), was depleted upon spheroplast formation, which sheds the peptidoglycan–arabinogalactan–mycomembrane complex, leaving a spherical cell with the plasma membrane intact (Figure S6).[50,51] This result indicated that neither probe was detected in the plasma membrane. Incorporation of N-x-AlkTMM-C15 was reduced when *Msmeg* was co-incubated with an unlabeled TMM competitor or the Ag85 inhibitor ebselen[52] (Figures S7 and S8). Furthermore, an Ag85 partial knockout mutant[53] exhibited reduced labeling by N-x-AlkTMM-C15 compared to a control peptidoglycan probe[54] (Figure S9). Collectively, these data demonstrate that photoactivatable TMM analogues incorporate into the native mycomembrane as anticipated.

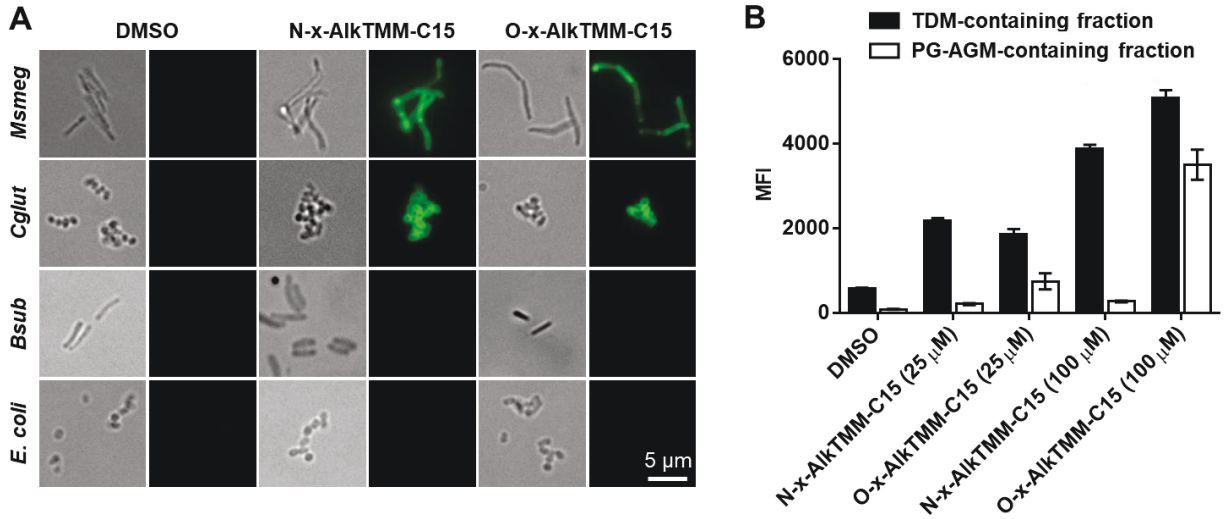


Figure 3. Mycomembrane labeling with N- and O-x-AlkTMM-C15. (A) Bacteria were cultured in probe (25 μM), reacted with azido-488 by CuAAC, and analyzed by microscopy (Figure S4, flow cytometry). (B) Probe-treated *Msmeg* was reacted with azido-488 by CuAAC, fractionated into PG-AGM- and TDM-containing fractions, and fluorescence was measured. Error bars denote the standard deviation of three replicates. MFI, mean fluorescence intensity in arbitrary units.

We next performed protein photo-crosslinking experiments in *Msmeg* using the TDM-targeting N-x-AlkTMM-C15 probe, which we prioritized primarily due to the complexities associated with O-x-AlkTMM-C15 labeling AGM (SI Discussion). N-x-AlkTMM-C15-treated live *Msmeg* was UV-irradiated, then lysates were collected, subjected to CuAAC with azido-488, and analyzed by SDS-PAGE. These experiments showed that proteins were labeled in a probe-, concentration-, and UV-dependent manner (Figure S10). *Msmeg* growth and metabolic activity were unaffected by UV irradiation (Figure S11), suggesting that UV-induced crosslinking in live cells occurred with minimal perturbation. To test whether N-x-AlkTMM-C15 photo-crosslinked our validation proteins, Ag85 and MspA, we enriched proteins and

performed Western blot analysis. Probe-treated *Msmeg* was UV-irradiated, then lysates were obtained and reacted with azido-TAMRA-PEG-biotin (AzTB) by CuAAC, delivering fluorescent and biotin tags to proteins for detection or enrichment. AzTB-treated lysates were analyzed by SDS-PAGE and Western blot prior to (input) and after (output) affinity capture on and elution from avidin beads. Proteins were effectively enriched only in the probe-treated, UV-irradiated (+probe+UV) samples (Figure 4A). Importantly, Ag85 and MspA were detected in all input samples, while both were clearly enriched in the outputs of the +probe+UV samples (Figure 4B and SI Discussion). The plasma membrane-associated mannosyltransferase MptA (negative control) was not detected in the outputs (Figure S12). These results show that N-x-AlkTMM-C15 enables photo-crosslinking, affinity enrichment, and detection of mycolate-interacting proteins.

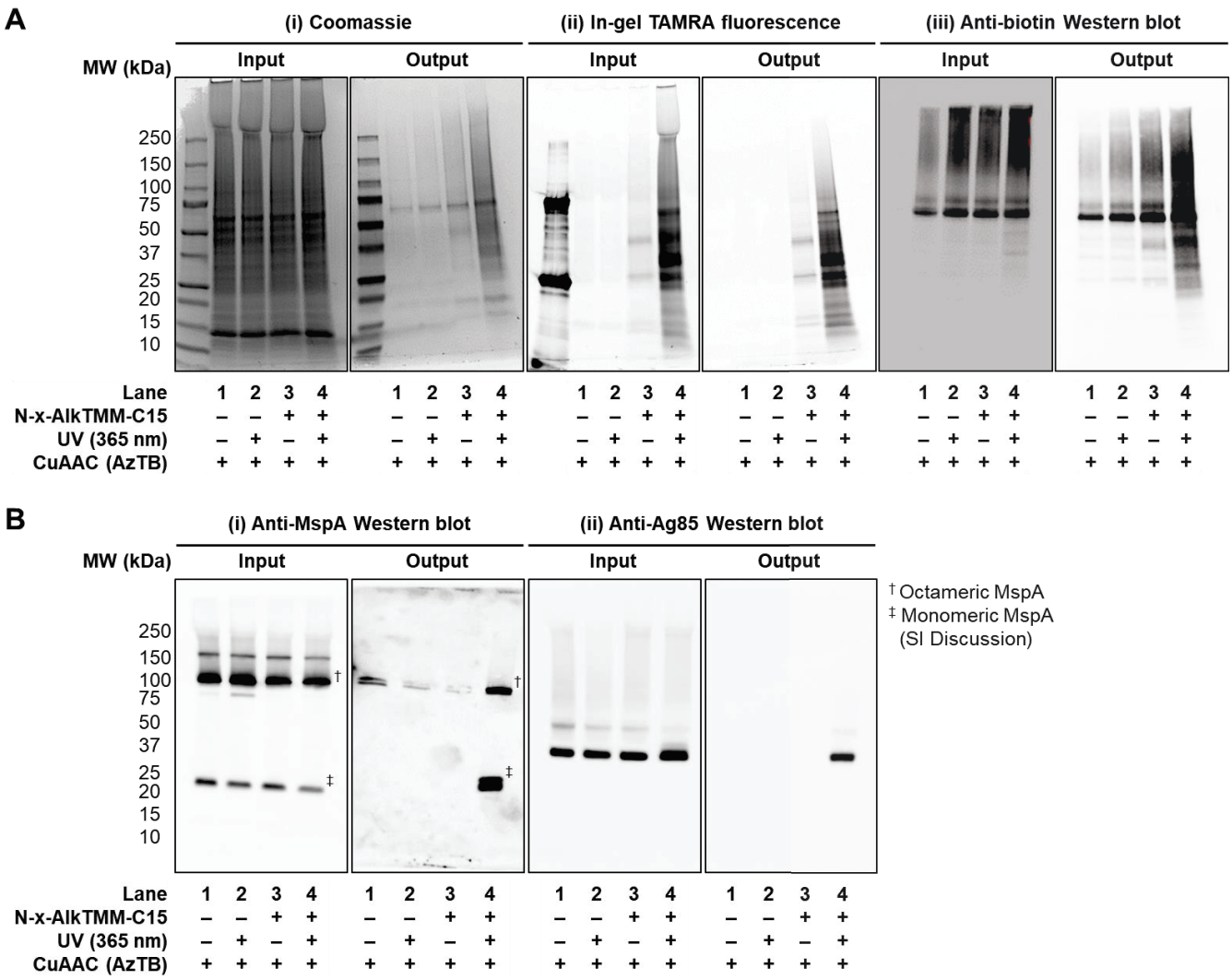


Figure 4. N-x-AlkTMM-C15-mediated affinity enrichment of mycolate-interacting proteins. *Msmeg* was cultured in N-x-AlkTMM-C15 (100 μM), UV-irradiated, and lysed. Lysates were reacted with AzTB by CuAAC, then analyzed using the indicated method before (input) and after (output) incubation with avidin beads to evaluate enrichment of (A) proteins in general and (B) MspA and Ag85. Data are representative of three independent experiments.

Finally, we used N-x-AlkTMM-C15 and label-free quantitative proteomics to identify mycolate–protein interactions in *Msmeg*, which was grown either for a shorter period to lower density (log phase) or an extended period to higher density (early stationary phase). In each of the two studies, LC–MS/MS analysis identified ~110 proteins that were significantly enriched by ≥4-fold in the +probe+UV group versus the +probe–UV control, of which ~75 proteins were identified exclusively in the +probe+UV group (Figure 5 and Tables S1–S4). These identifications included multiple Ag85 isoforms and MspA, consistent with Western blot analysis (Figure 4B) and confirming that N-x-AlkTMM-C15 photo-crosslinks mycolate-interacting proteins. Additional proteins with known mycolate-related functions were identified,

including CmrA, which is involved in TMM synthesis,[55,56] and MmpL3, which is the TMM flippase.[14,15] We identified multiple relevant hydrolases, including TDM hydrolase (Tdmh), which is involved in stress-induced mycomembrane remodeling,[57–59] and two related proteins, MSMEG_1528 and MSMEG_0194 (55% and 41% sequence identity to Tdmh), which potentially represent novel mycomembrane-remodeling enzymes. Other notable hits include EccA1, whose absence in *M. marinum* reduced mycolate synthesis by 40%,[60] and the extracellular proteins MTB12, MPT64, and HBHA, all of which have *Mtb* orthologs involved in host–pathogen interactions that are attractive diagnostic markers and/or vaccine candidates.[61–63] Indeed, most identified *Msmeg* proteins have *Mtb* orthologs, ~15–20% of which are essential *in vitro*,[64] and whose major predicted functions include cell wall/cell processes and uncharacterized hypothetical proteins (Tables S1 and S2; Figure S13). The differential protein profiles between our two studies, in terms of both protein identity and predicted functions (Figures 5C and S13), have interesting biological and experimental implications. The observed changes likely reflect a combination of growth phase-dependent dynamic changes in mycolate–protein interactions and of improved detection of low-abundance interactions in higher-density cultures (SI Discussion). Finally, the successful identification of nearly all known trehalose mycolate-interacting proteins in *Msmeg* (see Figure 1A) provides high confidence in probe specificity and thus in the biological relevance of the proteins identified through our strategy.

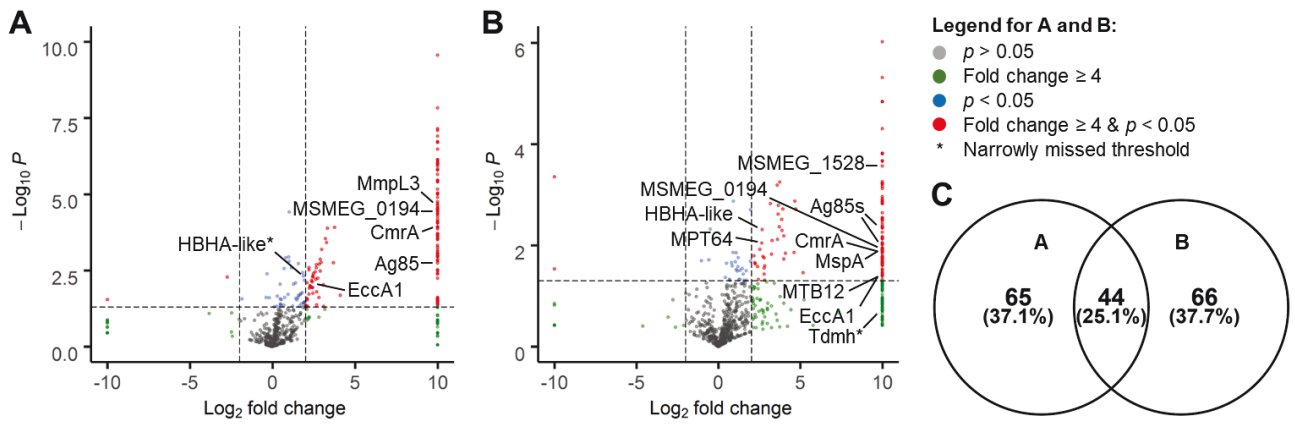


Figure 5. Volcano plots showing proteins in red that were significantly enriched in N-x-AlkTMM-C15-treated, UV-exposed (+probe+UV) versus non-irradiated (+probe–UV) *Msmeg* grown to OD_{600} (A) ~1.2 or (B) ~4 using click-mediated protein affinity enrichment, tryptic digestion, and LC–MS/MS analysis. Selected proteins of interest are indicated. (C) Venn diagram of proteins enriched in (A) and (B).

Given the importance of the mycomembrane to mycobacterial physiology and tuberculosis drug development, it is perplexing that such wide gaps in knowledge still exist with respect to its proteomic composition and the identities of proteins involved in its metabolism and host interactions. To date, the extraordinary complexity of the mycobacterial cell envelope—and the lack of suitable tools to experimentally dissect it—have impeded progress toward elucidating the structures and functions of mycomembrane-related proteins. As a new approach to solving this problem, we reported the first probes for capturing lipid–protein interactions in live mycobacteria and demonstrated their ability to identify mycolate-interacting proteins with known functions spanning mycomembrane synthesis, transport, and remodeling. We generated and analyzed protein lists containing numerous candidate mycolate interactors, many with unknown function, which—along with the probes themselves—are valuable for future research. Beyond expanding applications of N-x-AlkTMM-C15, we are further investigating O-x-AlkTMM-C15 and exploring a two-step approach using 6-TreAz[46] with our photoactivatable cyclooctynes.[48] Our tools' *in vivo* compatibility invites experimentation in diverse contexts (e.g., spatiotemporal proteomics, biofilms, infection models), while their generality motivates application to other mycobacteria, most importantly *Mtb*, which is labeled by N- and O-x-AlkTMM-C15 (Figure S14). Our approach can also be extended to study endogenous or host protein interactions with other types of mycobacterial lipids, which are widely appreciated for their distinctive structures and biological

importance. Ultimately, the ability to elucidate native-state lipid–protein interactions in mycobacteria will advance our understanding of mycobacterial physiology and pathogenesis, and may reveal new targets for the development of urgently needed tuberculosis vaccines, diagnostics, and drugs.

Supporting Information

Supplementary figures and schemes, supplementary discussion, experimental methods, and ^{1}H and ^{13}C NMR spectra. The Supporting Information is available free of charge on the ACS Publications website.

Corresponding Author

Benjamin M. Swarts, ben.swarts@cmich.edu

Acknowledgements

This work was supported by NSF CAREER Award 1654408 (B.M.S.), Camille and Henry Dreyfus Foundation Henry Dreyfus Teacher–Scholar Award TH-17-034 (B.M.S.), NIH R21 AI144748 (Y.S.M. and M.S.S.), NIH R03 AI140259-01 (Y.S.M.), NIH DP2 AI138238 (M.S.S.), and NIH R01 AI121354 (M.N.). LC–MS/MS was performed at the Michigan State Proteomics Facility by Dr. Douglas Whitten. We thank Dr. Kanna Palaniappan, Dr. Ben Luisi, and Dr. Amol Pohane for helpful discussions.

References

(1) World Health Organization, Global Tuberculosis Report 2019. https://www.who.int/tb/publications/global_report/en/

(2) Glaziou, P., Floyd, K., and Raviglione, M. C. (2018) Global Epidemiology of Tuberculosis. *Semin. Respir. Crit. Care Med. 39*, 271–285.

(3) Dheda, K., Gumbo, T., Gandhi, N. R., Murray, M., Theron, G., Udwadia, Z., Migliori, G. B., and Warren, R. (2014) Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. *Lancet Respir. Med. 2*, 321–338.

(4) Kurz, S. G., Furin, J. J., and Bark, C. M. (2016) Drug-Resistant Tuberculosis: Challenges and

Progress. *Infect. Dis. Clin. North Am. 30*, 509–522.

(5) Brennan, P. J. (2003) Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. *Tuberculosis 83*, 91–97.

(6) Angala, S. K., Belardinelli, J. M., Huc-Claustre, E., Wheat, W. H., and Jackson, M. (2014) The cell envelope glycoconjugates of Mycobacterium tuberculosis. *Crit. Rev. Biochem. Mol. Biol. 49*, 361–399.

(7) 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.

(8) Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G., and Daffe, M. (2008) Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *J. Bacteriol. 190*, 5672–5680.

(9) Barry, C. E., Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A., and Yuan, Y. (1998) Mycolic acids: Structure, biosynthesis and physiological functions. *Prog. Lipid Res. 37*, 143–179.

(10) Marrakchi, H., Lanéelle, M.-A., and Daffé, M. (2013) Mycolic acids: structures, biosynthesis, and beyond. *Chem. Biol. 21*, 67–85.

(11) Sani, M., Houben, E. N. G., Geurtsen, J., Pierson, J., de Punder, K., van Zon, M., Wever, B., Piersma, S. R., Jimenez, C. R., Daffe, M., Appelmelk, B. J., Bitter, W., van der Wel, N., Peters, P. J., Jiménez, C. R., Daffé, M., Appelmelk, B. J., Bitter, W., van der Wel, N., and Peters, P. J. (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS pathog. 6*, e1000794.

(12) North, E. J., Jackson, M., and Lee, R. E. (2014) New approaches to target the mycolic acid biosynthesis pathway for the development of tuberculosis therapeutics. *Curr. Pharm. Des. 20*, 4357–4378.

(13) Gavalda, S., Bardou, F., Laval, F., Bon, C., Malaga, W., Chalut, C., Guilhot, C., Mourey, L., Daffé, M., and Quémard, A. (2014) The Polyketide Synthase Pks13 Catalyzes a Novel Mechanism of Lipid Transfer in Mycobacteria. *Chem. Biol. 21*, 1660–1669.

(14) Grzegorzewicz, A. E., Pham, H., Gundi, V. A. K. B., Scherman, M. S., North, E. J., Hess, T.,

Jones, V., Gruppo, V., Born, S. E. M., 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.

(15) Xu, Z., Meshcheryakov, V. A., Poce, G., and Chng, S.-S. (2017) MmpL3 is the flippase for mycolic acids in mycobacteria. *Proc. Natl. Acad. Sci. U. S. A. 114*, 7993–7998.

(16) Sathyamoorthy, N., and Takayama, K. (1987) Purification and characterization of a novel mycolic acid exchange enzyme from Mycobacterium smegmatis. *J. Biol. Chem. 262*, 13417–13423.

(17) 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.

(18) Dautin, N., de Sousa-d'Auria, C., Constantinesco-Becker, F., Labarre, C., Oberto, J., de la Sierra-Gallay, I. L., Dietrich, C., Issa, H., Houssin, C., and Bayan, N. (2016) Mycoloyltransferases: A large and major family of enzymes shaping the cell envelope of Corynebacteriales. *Biochim. Biophys. Acta - Gen. Subj. 1861*, 3581–3592.

(19) Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C., and Engelhardt, H. (2010) Mycobacterial outer membranes: in search of proteins. *Trends Microbiol. 18*, 109–116.

(20) Pajon, R., Yero, D., Lage, A., Llanes, A., and Borroto, C. J. (2006) Computational identification of beta-barrel outer-membrane proteins in Mycobacterium tuberculosis predicted proteomes as putative vaccine candidates. *Tuberculosis 86*, 290–302.

(21) Song, H., Sandie, R., Wang, Y., Andrade-Navarro, M. A., and Niederweis, M. (2008) Identification of outer membrane proteins of Mycobacterium tuberculosis. *Tuberculosis 88*, 526–544.

(22) Mah, N., Perez-Iratxeta, C., and Andrade-Navarro, M. A. (2010) Outer membrane pore protein prediction in mycobacteria using genomic comparison. *Microbiology 156*, 2506–2515.

(23) Stahl, C., Kubetzko, S., Kaps, I., Seeber, S., and Engelhardt, H. (2001) MspA provides the main hydrophilic pathway through the cell wall of Mycobacterium smegmatis. *Mol. Microbiol. 40*, 451–464.

(24) Danilchanka, O., Sun, J., Pavlenok, M., Maueröder, C., Speer, A., Siroy, A., Marrero, J., Trujillo, C., Mayhew, D. L., Doornbos, K. S., Muñoz, L. E., Herrmann, M., Ehrt, S., Berens, C., and Niederweis, M. (2014) An outer membrane channel protein of Mycobacterium tuberculosis with exotoxin activity.

Proc. Natl. Acad. Sci. 111, 6750–6755.

(25) Speer, A., Rowland, J. L., Haeili, M., Niederweis, M., and Wolschendorf, F. (2013) Porins increase copper susceptibility of Mycobacterium tuberculosis. *J. Bacteriol. 195*, 5133–5140.

(26) Speer, A., Sun, J., Danilchanka, O., Meikle, V., Rowland, J. L., Walter, K., Buck, B. R., Pavlenok, M., Hölscher, C., Ehrt, S., and Niederweis, M. (2015) Surface hydrolysis of sphingomyelin by the outer membrane protein Rv0888 supports replication of Mycobacterium tuberculosis in macrophages. *Mol. Microbiol. 97*, 881–897.

(27) van der Woude, A. D., Mahendran, K. R., Ummels, R., Piersma, S. R., Pham, T. V, Jiménez, C. R., de Punder, K., van der Wel, N. N., Winterhalter, M., Luirink, J., Bitter, W., and Houben, E. N. G. (2013) Differential detergent extraction of Mycobacterium marinum cell envelope proteins identifies an extensively modified threonine-rich outer membrane protein with channel activity. *J. Bacteriol. 195*, 2050–2059.

(28) Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mailander, C., Engelhardt, H., and Niederweis, M. (2005) The growth rate of Mycobacterium smegmatis depends on sufficient porin-mediated influx of nutrients. *Mol. Microbiol. 58*, 714–730.

(29) Faller, M., Niederweis, M., and Schulz, G. E. (2004) The Structure of a Mycobacterial Outer-Membrane Channel. *Science 303*, 1189–1192.

(30) Wang, Q., Boshoff, H. I. M., Harrison, J. R., Ray, P. C., Green, S. R., Wyatt, P. G., and Barry, C. E. (2020) PE/PPE proteins mediate nutrient transport across the outer membrane of Mycobacterium tuberculosis. *Science 367*, 1147–1151.

(31) Korycka-Machala, M., Pawelczyk, J., Borowka, P., Dziadek, B., Brzostek, A., Kawka, M., Bekier, A., Rykowski, S., Olejniczak, A. B., Strapagiel, D., Witczak, Z., and Dziadek, J. (2020) PPE51 Is Involved in the Uptake of Disaccharides by Mycobacterium tuberculosis. *Cells 9*, E603. doi: 10.3390/cells9030603.

(32) Rezwan, M., Lanéelle, M. A., Sander, P., and Daffé, M. (2007) Breaking down the wall: Fractionation of mycobacteria. *J. Microbiol. Meth. 68*, 32–39.

(33) Marchand, C. H., Salmeron, C., Bou Raad, R., Méniche, X., Chami, M., Masi, M., Blanot, D., Daffé,

M., Tropis, M., Huc, E., Le Maréchal, P., Decottignies, P., and Bayan, N. (2011) Biochemical Disclosure of the Mycolate Outer Membrane of Corynebacterium glutamicum. *J. Bacteriol. 194*, 587–597.

(34) Chiaradia, L., Lefebvre, C., Parra, J., Marcoux, J., Burlet-Schiltz, O., Etienne, G., Tropis, M., and Daffé, M. (2017) Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Sci. Rep. 7*, 12807.

(35) He, Z., and Buck, J. De. (2010) Cell wall proteome analysis of Mycobacterium smegmatis strain MC2 155. *BMC Microbiol. 10*, 121.

(36) McNamara, M., Tzeng, S. C., Maier, C., Zhang, L., and Bermudez, L. E. (2012) Surface proteome of “Mycobacterium avium subsp. hominissuis” during the early stages of macrophage infection. *Infect. Immun. 80*, 1868–1880.

(37) Peng, T., Yuan, X., and Hang, H. C. (2014) Turning the spotlight on protein-lipid interactions in cells. *Curr. Opin. Chem. Biol. 21*, 144–153.

(38) Gubbens, J., Ruijter, E., Fays, L. E. V. De, Damen, J. M. A., Kruijff, B. De, Slijper, M., Rijkers, D. T. S., Liskamp, R. M. J., and Kroon, A. I. P. M. De. (2009) Photocrosslinking and Click Chemistry Enable the Specific Detection of Proteins Interacting with Phospholipids at the Membrane Interface. *Chem. Biol. 16*, 3–14.

(39) Haberkant, P., Raijmakers, R., Wildwater, M., Sachsenheimer, T., Brügger, B., Maeda, K., Houweling, M., Gavin, A.-C., Schultz, C., van Meer, G., Heck, A. J. R., and Holthuis, J. C. M. (2013) In Vivo Profiling and Visualization of Cellular Protein–Lipid Interactions Using Bifunctional Fatty Acids. *Angew. Chem. Int. Ed. 52*, 4033–4038.

(40) Hulce, J. J., Cognetta, A. B., Niphakis, M. J., Tully, S. E., and Cravatt, B. F. (2013) Proteome-wide mapping of cholesterol-interacting proteins in mammalian cells. *Nat. Methods 10*, 259.

(41) Sarkar, S., Libby, E. A., Pidgeon, S. E., Dworkin, J., and Pires, M. M. (2016) In Vivo Probe of Lipid II-Interacting Proteins. *Angew. Chem. Int. Ed. 55*, 8401–8404.

(42) Foley, H. N., Stewart, J. A., Kavunja, H. W., Rundell, S. R., and Swarts, B. M. (2016) Bioorthogonal chemical reporters for selective in situ probing of mycomembrane components in mycobacteria. *Angew. Chem. Int. Ed. 55*, 2053–2057.

(43) Kavunja, H. W., Piligian, B. F., Fiolek, T. J., Foley, H. N., Nathan, T. O., and Swarts, B. M. (2016) A chemical reporter strategy for detecting and identifying O-mycoloylated proteins in Corynebacterium. *Chem. Commun. 52*, 13795–13798.

(44) Fiolek, T. J., Banahene, N., Kavunja, H. W., Holmes, N. J., Rylski, A. K., Pohane, A. A., Siegrist, M. S., and Swarts, B. M. (2019) Engineering the Mycomembrane of Live Mycobacteria with an Expanded Set of Trehalose Monomycolate Analogues. *ChemBioChem 20*, 1282–1291.

(45) Hanessian, S., and Lavallee, P. (1972) Synthesis of 6-amino-6-deoxy-α,α-trehalose. Positional isomer of trehalosamine. *J. Antibiot. 25*, 683–684.

(46) Swarts, B. M., Holsclaw, C. M., Jewett, J. C., Alber, M., Fox, D. M., Siegrist, M. S., Leary, J. A., Kalscheuer, R., and Bertozzi, C. R. (2012) Probing the mycobacterial trehalome with bioorthogonal chemistry. *J. Am. Chem. Soc. 134*, 16123–16126.

(47) Sarpe, V. A., and Kulkarni, S. S. (2011) Synthesis of Maradolipid. *J. Org. Chem. 76*, 6866–6870.

(48) Stewart, J. A., Piligian, B. F., Rundell, S. R., and Swarts, B. M. (2015) A Trifunctional Cyclooctyne for Modifying Azide-Labeled Biomolecules with Photocrosslinking and Affinity Tags. *Chem. Commun. 51*, 17600–17603.

(49) Kuru, E., Hughes, H. V., Brown, P. J., Hall, E., Tekkam, S., Cava, F., de Pedro, M. A., Brun, Y. V, and VanNieuwenhze, M. S. (2012) In situ Probing of Newly Synthesized Peptidoglycan in Live Bacteria with Fluorescent D-Amino Acids. *Angew. Chem. Int. Ed. 51*, 12519–12523.

(50) Melzer, E. S., Sein, C. E., Chambers, J. J., and Siegrist, M. S. (2018) DivIVA concentrates mycobacterial cell envelope assembly for initiation and stabilization of polar growth. *Cytoskeleton 75*, 498–507.

(51) García-Heredia, A., Pohane, A. A., Melzer, E. S., Carr, C. R., Fiolek, T. J., Rundell, S. R., Chuin Lim, H., Wagner, J. C., Morita, Y. S., Swarts, B. M., and Siegrist, M. S. (2018) Peptidoglycan precursor synthesis along the sidewall of pole-growing mycobacteria. *Elife 7*, e37243.

(52) Favrot, L., Grzegorzewicz, A. E., Lajiness, D. H., Marvin, R. K., Boucau, J., Isailovic, D., Jackson, M., and Ronning, D. R. (2013) Mechanism of inhibition of Mycobacterium tuberculosis antigen 85 by ebselen. *Nat. Commun. 4*, 2748.

(53) Kamariza, M., Shieh, P., Ealand, C. S., Peters, J. S., Chu, B., Rodriguez-Rivera, F. P., Babu Sait, M. R., Treuren, W. V, Martinson, N., Kalscheuer, R., Kana, B. D., and Bertozzi, C. R. (2018) Rapid detection of Mycobacterium tuberculosis in sputum with a solvatochromic trehalose probe. *Sci. Transl. Med. 10*, eaam6310.

(54) Siegrist, M. S., Whiteside, S., Jewett, J. C., Aditham, A., Cava, F., and Bertozzi, C. R. (2013) d-Amino Acid Chemical Reporters Reveal Peptidoglycan Dynamics of an Intracellular Pathogen. *ACS Chem. Biol. 8*, 500–505.

(55) Lea-Smith, D. J., Pyke, J. S., Tull, D., McConville, M. J., Coppel, R. L., and Crellin, P. K. (2007) The reductase that catalyzes mycolic motif synthesis is required for efficient attachment of mycolic acids to arabinogalactan. *J. Biol. Chem. 282*, 11000–11008.

(56) Bhatt, A., Brown, A. K., Singh, A., Minnikin, D. E., and Besra, G. S. (2008) Loss of a mycobacterial gene encoding a reductase leads to an altered cell wall containing beta-oxo-mycolic acid analogs and accumulation of ketones. *Chem. Biol. 15*, 930–939.

(57) 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.

(58) Yang, Y., Kulka, K., Montelaro, R. C., Reinhart, T. A., Sissons, J., Aderem, A., and Ojha, A. K. (2014) A Hydrolase of Trehalose Dimycolate Induces Nutrient Influx and Stress Sensitivity to Balance Intracellular Growth of Mycobacterium tuberculosis. *Cell Host Microbe 15*, 153–163.

(59) Holmes, N. J., Kavunja, H. W., Yang, Y., Vannest, B. D., Ramsey, C. N., Gepford, D. M., Banahene, N., Poston, A. W., Piligian, B. F., Ronning, D. R., Ojha, A. K., and Swarts, B. M. (2019) A FRET-Based Fluorogenic Trehalose Dimycolate Analogue for Probing Mycomembrane-Remodeling Enzymes of Mycobacteria. *ACS Omega 4*, 4348–4359.

(60) Joshi, S. A., Ball, D. A., Sun, M. G., Carlsson, F., Watkins, B. Y., Aggarwal, N., McCracken, J. M., Huynh, K. K., and Brown, E. J. (2012) EccA1, a component of the Mycobacterium marinum ESX-1 protein virulence factor secretion pathway, regulates mycolic acid lipid synthesis. *Chem. Biol. 19*, 372–380.

(61) Lee, J.-S., Son, J. W., Jung, S.-B., Kwon, Y.-M., Yang, C.-S., Oh, J.-H., Song, C.-H., Kim, H.-J., Park, J.-K., Paik, T.-H., and Jo, E.-K. (2006) Ex vivo responses for interferon-gamma and proinflammatory cytokine secretion to low-molecular-weight antigen MTB12 of Mycobacterium tuberculosis during human tuberculosis. *Scand. J. Immunol. 64*, 145–154.

(62) Wang, Q., Liu, S., Tang, Y., Liu, Q., and Yao, Y. (2014) MPT64 protein from Mycobacterium tuberculosis inhibits apoptosis of macrophages through NF-kB-miRNA21-Bcl-2 pathway. *PLoS One 9*, e100949–e100949.

(63) Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C., and Menozzi, F. D. (2001) The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. *Nature 412*, 190–194.

(64) DeJesus, M. A., Gerrick, E. R., Xu, W., Park, S. W., Long, J. E., Boutte, C. C., Rubin, E. J., Schnappinger, D., Ehrt, S., Fortune, S. M., Sassetti, C. M., and Ioerger, T. R. (2017) Comprehensive Essentiality Analysis of the Mycobacterium tuberculosis Genome via Saturating Transposon Mutagenesis. *MBio 8*, e02133-16.