<u>**Supporting Information**</u>.

Phylogenetic and biochemical analyses of mycobacterial L,D-transpeptidases reveal a distinct enzyme class that is preferentially acylated by meropenem.

Trevor A. Zandi[1], Robert L. Marshburn[2], Paige K. Stateler[2], Leighanne A. Brammer Basta[2]*

[1]Department of Chemistry, Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218
[2]Chemistry Department, United States Naval Academy, 572M Holloway Rd., Annapolis, MD 21402

***Corresponding author**: Email: basta@usna.edu; Telephone: (410) 293-6603

Table S1. Summary of *Msm* Ldt proteins used in this study.

Table S2. Summary of primers used in site-directed mutagenesis reactions.

Figure S1. Complete phylogenetic tree of examined mycobacterial Ldts. See attached file.

Figure S2. Conserved regions found in the catalytic domains of class 1 and 3 Ldts

Figure S3. Comparison of mycobacterial class 1 and class 3 Ldt catalytic domains.

Figure S4. Analysis of class 4 Ldts.

Figure S5. Classification of mycobacterial species examined in this study.

Figure S6. *Msm* LdtC acylation by (carba)penems.

Figure S7. Homology models of LdtB and LdtF with docked (carba)penems.

Figure S8. UPLC-MS data of LdtF D353W variant.

Figure S9. UPLC-MS data of LdtF D353N and D353A variants.

Figure S10. LdtB competition experiment.

Protein	Actual construct
LdtA	Δ^{1-49}LdtA
LdtB	Δ^{1-26}LdtB
LdtC	Δ^{1-48}LdtC
LdtD (formerly LdtG)	Δ^{1-21}LdtD
LdtE	Δ^{1-22}LdtE
LdtF	Δ^{1-33}LdtF

Table S1. Summary of *Msm* Ldt proteins used in this study.

***Msm* LdtF variant**	**Primers**
D353A	F: 5′- CTGCACCAGCTCAACGCCACCATCTGGGCGCAG-3′ R: 5′- CTGCGCCCAGATGGTGGCGTTGAGCTGGTGCAG-3′
D353N	F: 5′- CTGCACCAGCTCAACAACACCATCTGGGCGCAG-3′ R: 5′- CTGCGCCCAGATGGTGTTGTTGAGCTGGTGCAG-3′
D353W	F: 5′- CCTGCACCAGCTCAACTGGACCATCTGGGCGCAGG-3′ R: 5′- CCTGCGCCCAGATGGTCCAGTTGAGCTGGTGCAGG-3′

Table S2. Summary of primers used to generate *Msm* LdtF variants. F, forward primer; R, reverse primer.

Figure S1. Complete phylogenetic tree of examined mycobacterial Ldts.

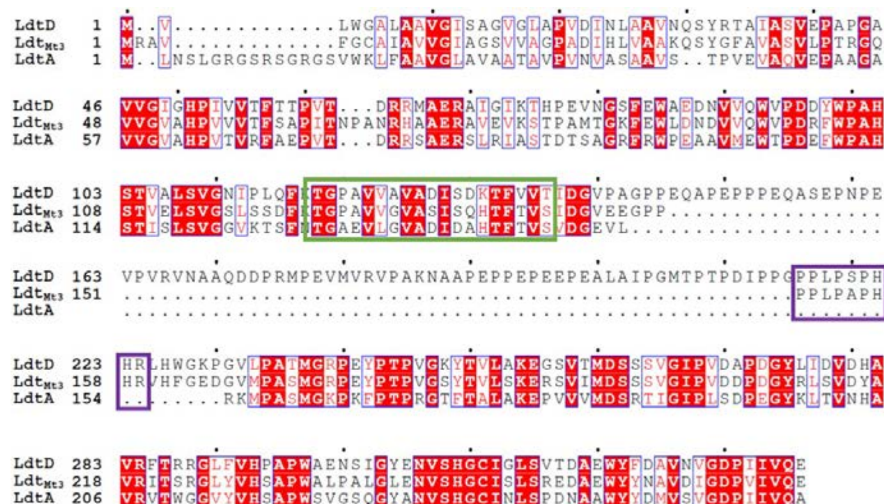

Figure S2. Class 3 Ldts contain Region 2 sequences of varying lengths. Region 2 of *Msm* LdtD is *N*-terminally extended and rich in proline and glutamate compared to Region 2 of *Mtb* $\mathrm{Ldt_{Mt3}}$[1] (boxed in purple). *Msm* LdtA (class 1) lacks Region 2. Region 1 is boxed in green and is present in class 1 and 3 Ldts. Alignment was performed and visualized using Clustal Omega and ESPript 3.0, respectfully.[2,3]

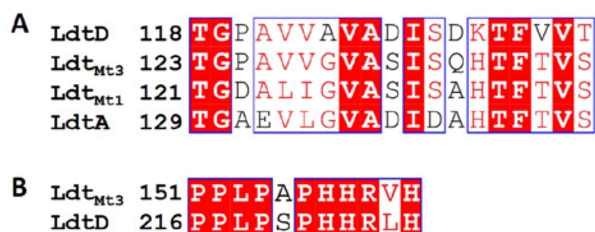

Figure S3. Conserved regions found in the catalytic domains (CDs) of class 1 and 3 Ldts. **A**) Region 1 is present in both class 1 (*Msm* LdtA and *Mtb* $\mathrm{Ldt_{Mt1}}$ from *Mtb*) and class 3 Ldts (Msm LdtD (formerly LdtG) and *Mtb* $\mathrm{Ldt_{Mt3}}$), while **B**) Region 2 is present in only class 3 Ldts. Neither of these regions are present in class 2, 4, 5 or 6 Ldts. Alignment was performed and visualized using Clustal Omega and ESPript 3.0, respectfully.[2,3]

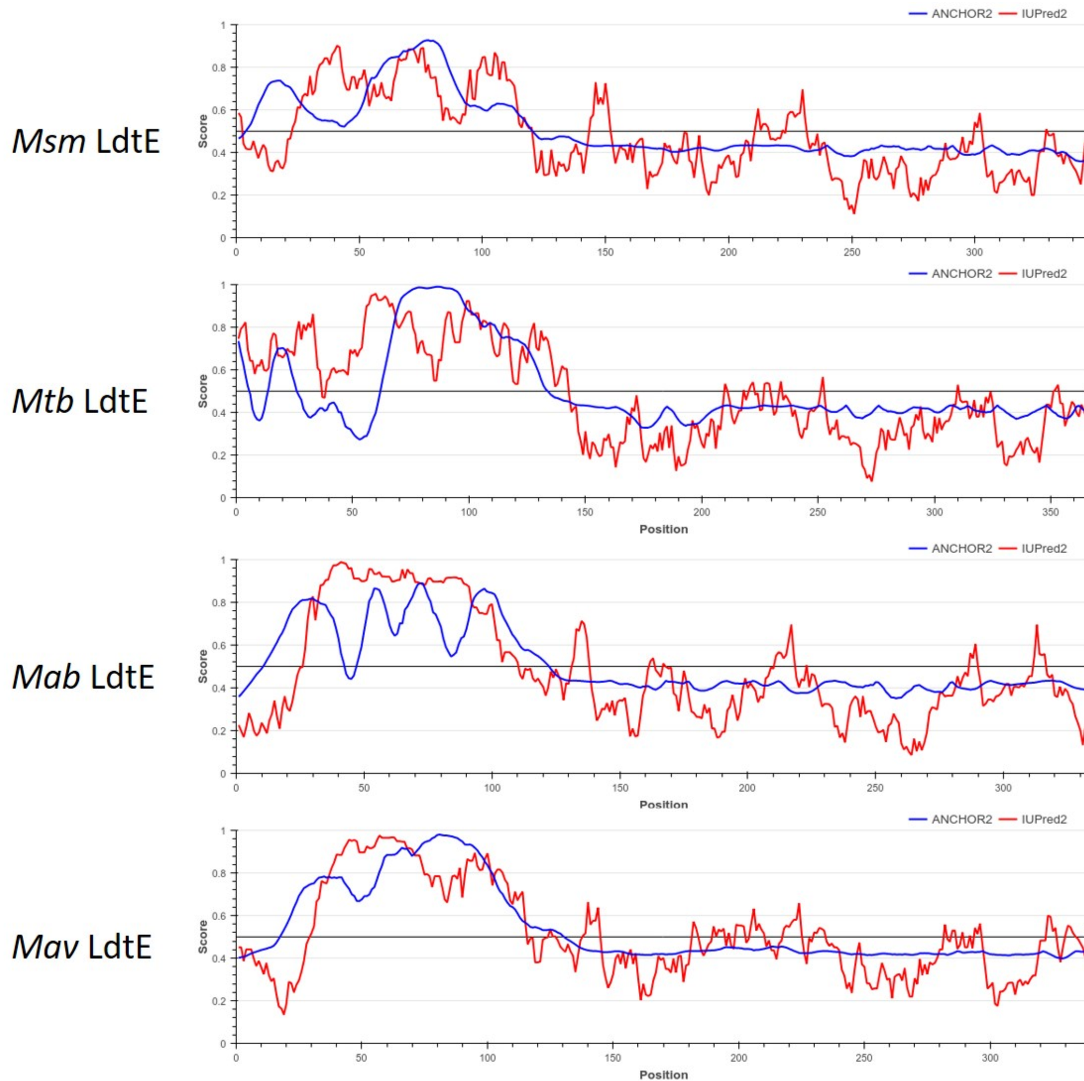

Figure S4. The *N*-terminus of class 4 Ldts is likely intrinsically disordered and mediates a protein-protein interaction. IUPred2a (red) depicts probability of each residue being in a disordered region as a score between 0 and 1, where residues with scores greater than 0.5 are predicted to be disordered. ANCHOR2 (blue) depicts probability of each residue being part of a disordered region that binds a protein, where residues with scores greater than 0.5 are predicted to be disordered protein binding regions.[4] *Msm* = *Mycobacterium smegmatis*, *Mtb* = *Mycobacterium tuberculosis*, *Mab* = *Mycobacterium abscessus*, *Mav* = *Mycobacterium avium*.

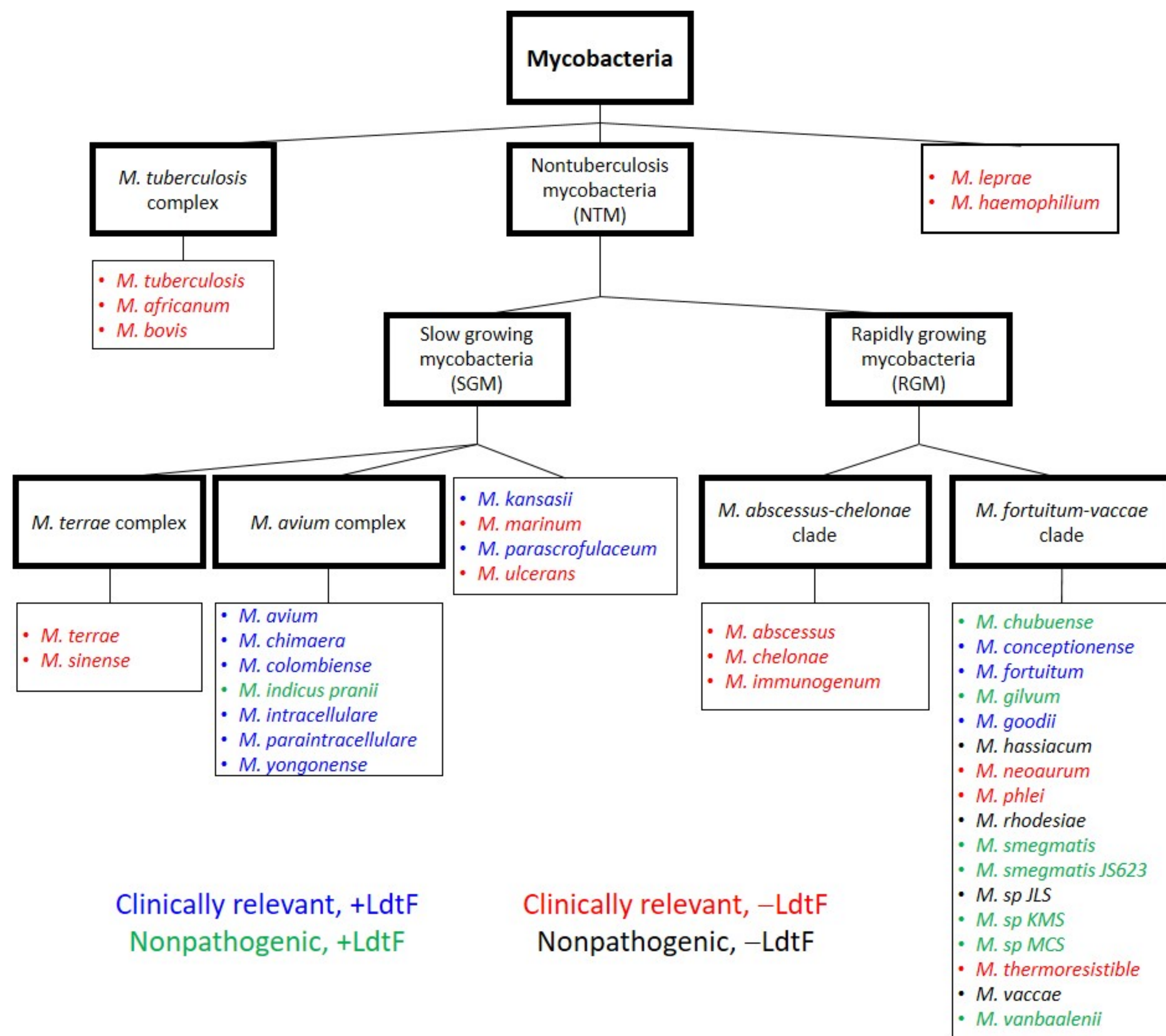

Figure S5. Classification of mycobacteria relevant to this study.[5] Species in red and blue are clinically relevant, as they are pathogenic or can cause infections, nosocomial or otherwise. Those in green and black are nonpathogenic and either do or do not express LdtF, respectively.

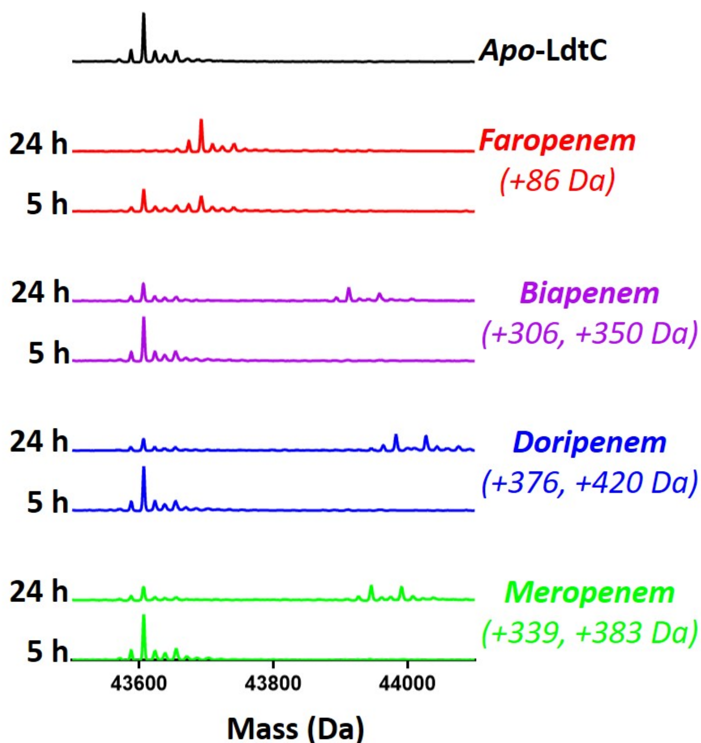

Figure S6. *Msm* LdtC is fully acylated by faropenem after 24 hours. Appreciable amounts of other adducts are not detected after 5 hours, but LdtC is partially acylated by biapenem, doripenem, and meropenem after 24 hours.

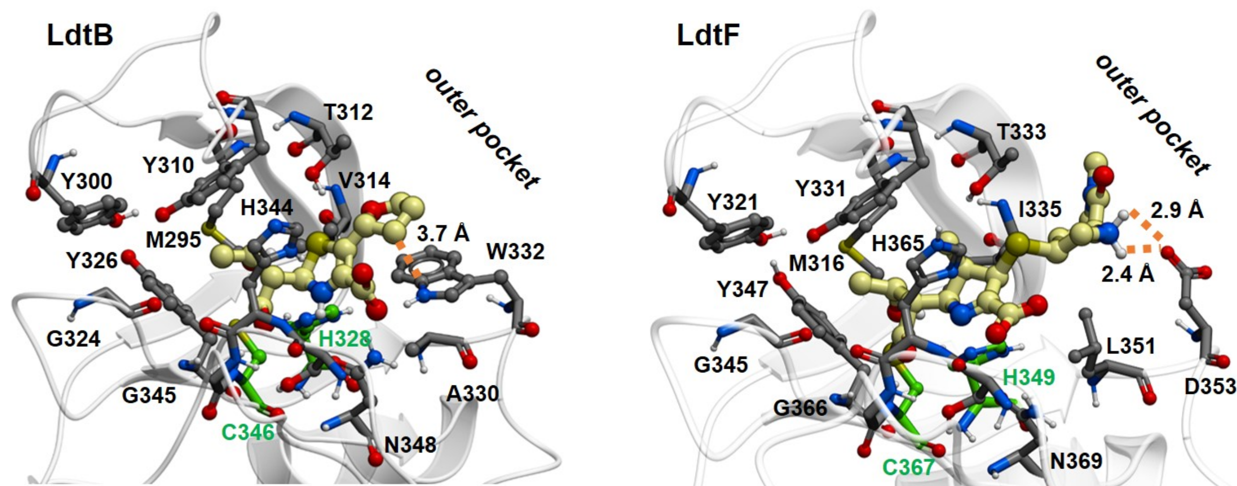

Figure S7. A conserved tryptophan residue (Trp332, left panel) is replaced with an aspartate (Asp353) in LdtF (right panel). Catalytic residues cysteine and histidine are colored green. Faropenem (left) and meropenem (right) are shown as yellow balls and sticks.

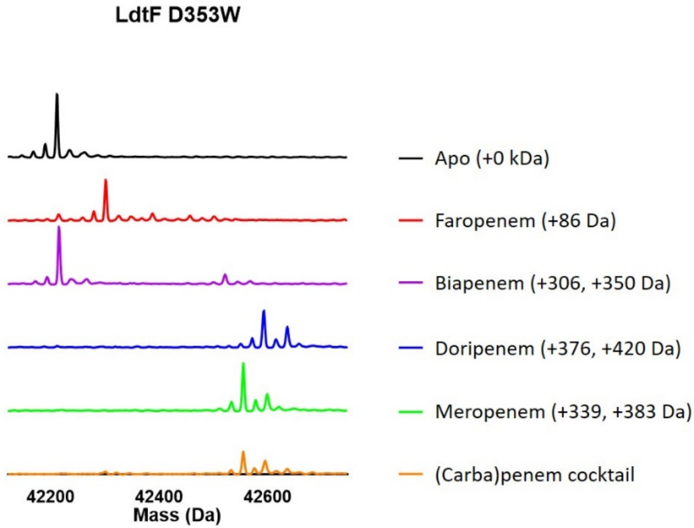

Figure S8. LdtF D353W variant is preferentially acylated by meropenem in a competition experiment.

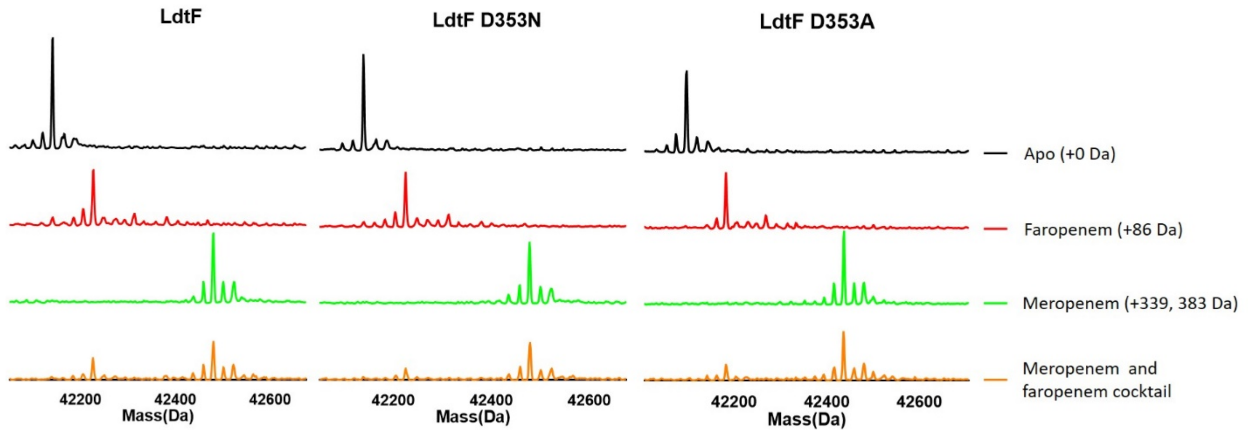

Figure S9. LdtF D353N and D353A variant UPLC-MS profiles following incubation with faropenem, meropenem, or both drugs at equal concentrations. Meropenem preferentially acylates the LdtF variants.

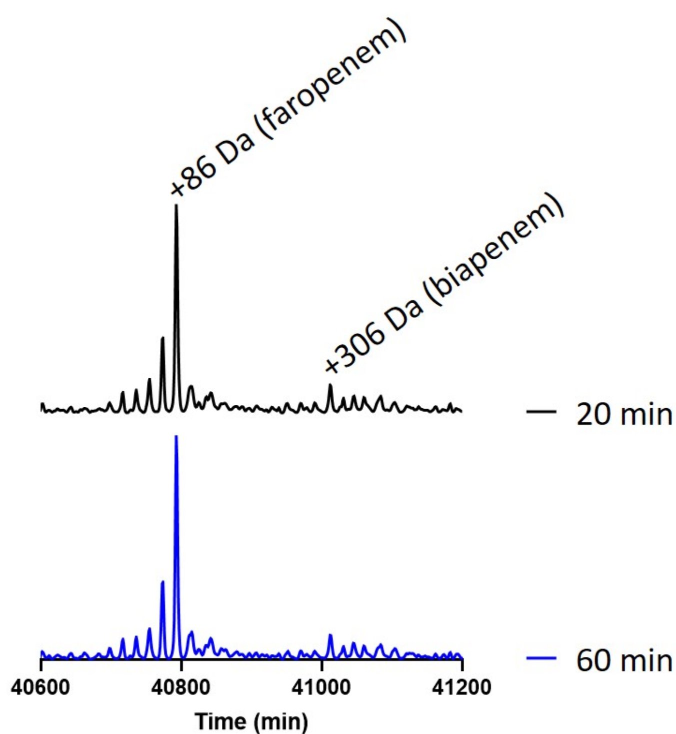

Figure S10. *Msm* LdtB is fully acylated by faropenem after 20 minutes when the drug is provided as a cocktail with equal molar amounts of biapenem, doripenem, and meropenem.

Methods

Site-directed Mutagenesis Studies. Site-directed mutagenesis reactions were performed as described previously with minor modifications.[6] Briefly, two polymerase chain reactions (25 µL volume) containing either the forward or the reverse primer were set up in parallel. Each reaction contained 1× GC reaction buffer (New England Biolabs), 200 µM dNTPs, 3% DMSO, 20 ng/µL template DNA, 1 U of Phusion polymerase (New England Biolabs) and 500 µM of either the forward or reverse primer. The ldtF-pET28b plasmid was used as template to generate each LdtF variant (Table 2). A two-step PCR protocol was used (denaturation, 30 sec at 98 °C; annealing and extension, 4 min at 72 °C) because of the high T_m of the primers used. Forward and reverse PCRs were combined (new volume of 50 µL) and complementary strands were reannealed by heating and slowly cooling the reactions (95 °C, 5 min; 90 °C, 1 min; 80 °C, 1 min; 70 °C, 30 sec; 60 °C, 30 sec; 50 °C, 30 sec; 40 °C, 30 sec). The temperature of the reaction was held at 37 °C and the template was digested with Dpn1 (3 U, 4 hours). All constructs were fully sequenced. Competent bacteria were transformed with the plasmids and Msm variants were purified as described in the main text of this manuscript.

References

(1) Sanders, A. N.; Wright, L. F.; Pavelka, M. S. Genetic Characterization of Mycobacterial L,D-Transpeptidases. *Microbiol. (United Kingdom)* **2014**, *160*, 1795–1806. doi.org/10.1099/mic.0.078980-0.

(2) Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T. J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Soding, J.; et al. Fast, Scalable Generation of High-Quality Protein Multiple Sequence Alignments Using Clustal Omega. *Mol Syst Biol* **2011**, *7*, 539. doi.org/10.1038/msb.2011.75.

(3) Robert, X.; Gouet, P. Deciphering Key Features in Protein Structures with the New ENDscript Server. *Nucleic Acids Res.* **2014**, *42* (W1), W320–W324. doi.org/10.1093/nar/gku316.

(4) Mészáros, B.; Erdös, G.; Dosztányi, Z. IUPred2A: Context-Dependent Prediction of Protein Disorder as a Function of Redox State and Protein Binding. *Nucleic Acids Res.* **2018**. doi.org/10.1093/nar/gky384.

(5) Gupta, R. S.; Lo, B.; Son, J. Phylogenomics and Comparative Genomic Studies Robustly Support Division of the Genus *Mycobacterium* into an Emended Genus *Mycobacterium* and Four Novel Genera. *Front Microbiol* **2018**, *9*, 67. doi.org/10.3389/fmicb.2018.00067.

(6) Brammer Basta, L. A.; Ghosh, A.; Pan, Y.; Jakoncic, J.; Lloyd, E. P.; Townsend, C. A.; Lamichhane, G.; Bianchet, M. A. Loss of a Functionally and Structurally Distinct Ld-Transpeptidase, Ldt_{Mt5}, Compromises Cell Wall Integrity in *Mycobacterium tuberculosis*. *J. Biol. Chem.* **2015**, *290* (42), 25670–25685. doi.org/10.1074/jbc.M115.660753.