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Investigating β -Lactam Drug Targets in *Mycobacterium tuberculosis* Using Chemical Probes

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Abstract

Tuberculosis (TB), caused by the bacterial pathogen *Mycobacterium tuberculosis* (*Mtb*), infects 10 million people a year. An estimated 25% of humans harbor latent TB infections, an asymptomatic form of the disease. In both active and latent infections, *Mtb* relies on cell wall peptidoglycan for viability. In the current work, we synthesized fluorescent analogues of β -lactam antibiotics to study two classes of enzymes that maintain *Mtb*'s peptidoglycan: penicillin-binding proteins (PBPs) and L,D -transpeptidases (LDTs). This set of activity-based probes included analogues of three classes of β -lactams: a monobactam (aztreonam-Cy5), a cephalosporin (cephalexin-Cy5), and a carbapenem (meropenem-Cy5). We used these probes to profile enzyme activity in protein gel-resolved lysates of *Mtb*. All three out-performed the commercial reagent Bocillin-FL, a penam. Meropenem-Cy5 was used to identify β -lactam targets by mass spectrometry, including PBPs, LDTs, and the β -lactamase BlaC. New probes were also used to compare PBP and LDT activity in two metabolic states: dormancy and active replication. We provide the first direct evidence that *Mtb* dynamically regulates the enzymes responsible for maintaining peptidoglycan in dormancy. Lastly, we profiled drug susceptibility in lysates and found that meropenem inhibits PBPs, LDTs, and BlaC.

Graphical Abstract

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Supporting Information

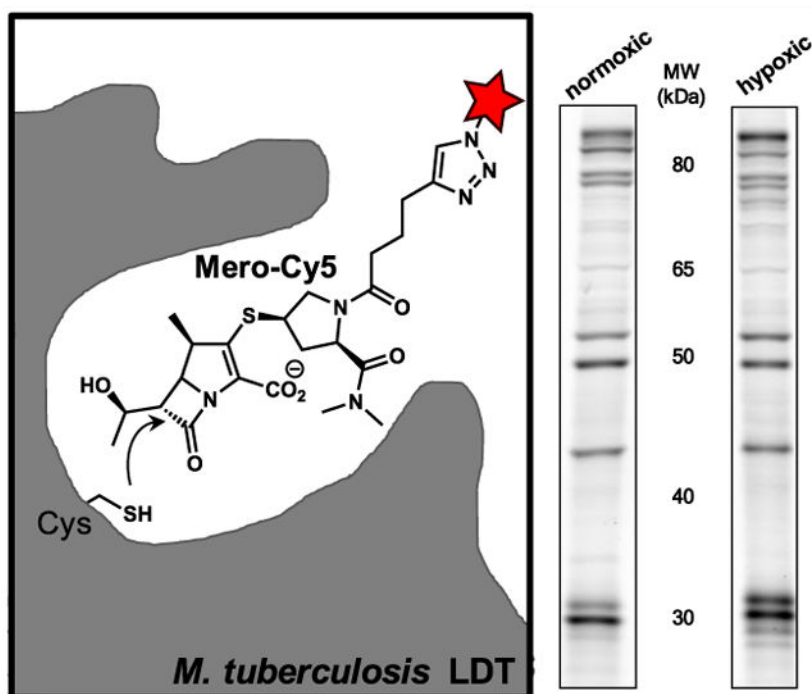
The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscinfecdis.0c00809>.

Tables of enzyme list and band excision data (XLSX)

Discussions of characterization and synthetic methods used, Cu-catalyzed azide-alkyne click reaction, mycobacterial culture conditions, hypoxia induced dormancy, preparation of lysates, analysis of labeled lysates by SDS-PAGE, detection of phosphoproteins in SDS-PAGE gels, and band excision for LC-MS/MS-based proteomics and figures of SDS-PAGE gels, Bocillin-FL labeling, Images of gels used for band excision experiments, visualization of the division of protein gels, protein identifications for bands, analysis of lysates, identification of proteins associated with fluorescent bands, analysis of the phosphorylation state of proteins labeled with Mero-Cy5, β -lactam probes show differences in transpeptidase activity, Azt-Cy5 and CephX-Cy5 selectively label proteins, LC trace and LC-MS results, and ^1H NMR spectra (PDF)

Raw data files, results, peak lists, and search engine files for proteomics experiments will be deposited in an online database (i.e., ProteomeXchange via MassIVE).

The authors declare no competing financial interest.



Keywords

antibiotic; tuberculosis; activity-based probe; fluorescent; carbapenem; proteomics

Mycobacterium tuberculosis (*Mtb*) has the ignominious distinction of being the deadliest bacterial pathogen in the world. It causes both active tuberculosis (TB) and asymptomatic, latent disease.^{1,2} Latent TB infections (LTBIs) are common and widespread, creating a substantial barrier to eradication. In the human host, *Mtb* can transition to a dormant, non-replicating state in response to hypoxia, nutrient starvation, or other harsh conditions.³⁻⁵ Dormant *Mtb* is resistant to treatment with most front-line drugs.^{6,7} While dormant *Mtb* is associated with LTBIs, active TB patients also harbor metabolically varied *Mtb*.^{1,8,9} This suggests that the most effective drugs for curing TB will target both actively replicating and dormant *Mtb*.

Regardless of the metabolic state, maintenance of the cell wall peptidoglycan (PG) is required for *Mtb* viability. PG is composed of glycan chains linked to peptide stems that are cross-linked to form a mesh that surrounds bacterium.¹⁰ Two of the earliest classes of β -lactam drugs, the penicillins and cephalosporins, inhibit D,D-transpeptidases (DDTs). These enzymes are aptly called penicillin-binding proteins (PBPs), and they covalently modify PG.¹¹ DDTs form 4 \rightarrow 3 cross-links between a stem peptide's fourth amino acid, D-alanine, and the third amino acid on an adjacent stem: meso-diaminopimelic acid (mDAP, Figure 1). In most bacteria, the PG is composed of 4 \rightarrow 3 linkages. Yet in *Mtb*, 3 \rightarrow 3 cross-links compose up to 80% of linkages.^{12,13} This covalent modification, bonding between mDAP³ residues, is made by L,D-transpeptidases (LDTs). Both LDTs and PBPs are critical to

survival because they maintain the structure and rigidity of PG. The *Mtb* LDTs and PBPs are summarized in Table 1.

Mtb uses PBPs, LDTs, and other enzymes to modify cell wall PG to survive environmental changes.^{14,15} Transcriptional analyses support that the PBPs and LDTs are dynamically regulated during these metabolic transitions. The *Ldt_{Mt1}* transcript is up-regulated 17-fold under nutrient starvation, while PBPA is down-regulated.³ *DacB1*'s transcript is up-regulated 5-fold under conditions that induce dormancy.¹⁶ The corresponding enzymes likely have non-redundant roles in dormancy, and PBPs and LDTs are biochemically distinct enzyme classes.¹¹ For example, PBPA is an essential enzyme,¹⁷ and both *DacB1* and *PonA2* are required for infectivity.^{18,19} *PonA2*, a bifunctional transglycosylase and DDT, is associated with non-replicating mycobacteria²⁰ and survival under hypoxia.²¹ The loss of *PonA2* confers β -lactam drug-resistance and makes *Ldt_{Mt2}* essential.²² *Ldt_{Mt1}* and *Ldt_{Mt2}* are required for virulence,^{23,24} and *Ldt_{Mt1}* is believed to remodel PG for survival in dormancy.^{3,12} There is recent evidence that LDTs mediate PG maturation and confer cell shape in mycobacteria.^{25,26}

The central role of LDTs and PBPs in *Mtb* PG biosynthesis and infection makes them candidate drug targets for treating TB. β -lactam antibiotics target PG biosynthesis and are widely used, safe, and effective drugs. However, they are not part of the standard treatment regimen for TB. One reason for this is likely historical.²⁷ A study from 1949 described a "penicillinase" that made *Mtb* resistant to β -lactam antibiotics.²⁸ The enzyme responsible is *BlaC*, a β -lactamase, and its discovery indicated that those first β -lactam antibiotics would be unsuccessful in treating TB. *BlaC* also inactivated the cephalosporin class, which was introduced in the 1960s.²⁹ Yet, we now know that *BlaC* has limited activity against carbapenems³⁰ and is inactivated by clavulanate,³¹ a clinically approved β -lactamase inhibitor. Moreover, as reviewed by Story-Roller and Lamichhane,²⁷ the carbapenem class inhibits LDTs, which may provide distinct (non-PBP) drug targets in *Mtb*.

To summarize, the discovery of the mycobacterial LDTs^{12,32} has renewed interest in understanding how PBPs and LDTs maintain PG integrity and how these enzymes interact with β -lactam antibiotics. A central motivation for this work is the potential for using clinically approved β -lactams to treat TB.^{22,27,33} However, the scarcity of information on protein targets of β -lactams in *Mtb* makes the development of new treatment regimens challenging. Furthermore, there is almost no information on which PBPs and LDTs are active in dormant *Mtb*.

Evaluating PBPs and LDTs together would be challenging using conventional biochemical or genetic approaches. A different option is to use activity-based probes (ABPs), which facilitate monitoring of entire classes of enzymes at once in lysates or tissues. ABPs consist of a reporter linked to an irreversible inhibitor, which provides specificity for the enzyme target.^{34,35} There is an established precedence for using ABPs to assign peptidase functions in other bacterial species.³⁶⁻⁴¹ Although both the PBPs and LDTs are susceptible to inhibition by β -lactam antibiotics, their substrate preferences are distinct. For example, PBPs are inactivated by covalent modification at a catalytic serine by various β -lactam antibiotics (e.g., penams, monobactams, cepheids, and carbapenems).⁴²⁻⁴⁴ In contrast, LDTs are

inhibited by compounds in the carbapenem class, which bind to an active-site cysteine by thioester bond formation (Figure 2).^{12,32,45–47} Characterizing the targets of β -lactam antibiotics in *Mtb* would therefore require ABPs based on carbapenems and other scaffolds.

In this work, we used ABPs to profile the activity and regulation of *Mtb* PBPs and LDTs. We produced a small set of structurally diverse β -lactam probes, including a new fluorescent meropenem. We used these probes to analyze PBPs and LDTs in protein gel-resolved mycobacterial lysates. We identified enzymes associated with both dormant and actively replicating *Mtb*. Lastly, our results show that carbapenems, including meropenem, inhibit many of the *Mtb* PBPs and LDTs, suggesting that this class of β -lactam could be useful for treating latent or active TB infections.

RESULTS AND DISCUSSION

Identification of β -Lactam Targets Encoded in the *Mtb* Genome.

We initiated this project by generating a comprehensive list of *Mtb* enzymes with active sites likely to interact with a β -lactam. We searched the *Mtb* H37Rv genome for genes with protein family (i.e., Pfam) annotation indicating PBP, LDT, or β -lactamase activity.⁴⁸ This search gave a total of 43 proteins with either validated or theoretical abilities to interact with β -lactams (see Supporting Information, Table S1). The abbreviated version of this list, Table 1, contains only the most likely β -lactam targets and includes 11 PBPs and five LDTs. This list was generated by combining the PBPs identified by Sauvage et al.,⁴⁹ the five known LDTs,¹¹ and BlaC.

Synthesis and Validation of Fluorescent β -Lactam Probes.

Next, we assembled our chemical toolkit for analyzing β -lactam targets in *Mtb* (Figure 3). For our studies, all ABPs contained a β -lactam antibiotic as the enzyme inhibitor. We obtained Bocillin-FL to represent the penam class, which is a commercially available green fluorescent penicillin.³⁶ We additionally selected monobactam, cephalosporin, and carbapenem scaffolds for structural diversity. An alkyne handle was included in these ABPs to enable conjugation to fluorescent probes *via* a copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC). In designing the probes, we modified parent compounds at a position amenable to the synthetic introduction of an alkyne. Additionally, we selected a location that would be unlikely to disrupt binding. For example, we introduced the alkyne at a position in meropenem that was expected to orient the reporter out of the LDT active site.⁴⁷ Lastly, we opted for probes derived from clinically approved β -lactam antibiotics.

For the monobactam compound, we synthesized the known aztreonam-*N*-alkyne.⁴⁰ We found that increasing the reaction time resulted in a substantially higher yield than was previously reported (>95% yield in 3 d vs 19% in 30 min).⁴⁰ For the cephalosporin and carbapenem scaffolds, we synthesized two new compounds, cephalixin-alkyne and meropenem-alkyne,⁵⁰ respectively. For the cephalosporin probe, we used the readily available cephalixin as the starting material, providing a cost benefit over the more traditional cephalosporin C.^{38,40} We synthesized cephalixin-alkyne under basic conditions similar to ones used by Carlson and co-workers to synthesize cephalosporin C probes.³⁸ A

modification of these conditions to use a heterogeneous base (Cs_2CO_3) generated meropenem-alkyne from meropenem. We selected meropenem as our carbapenem-class probe because of its clinical significance for treating TB.⁵¹ Also, it was straightforward to modify, unlike faropenem and imipenem. These alkynes readily underwent CuAAC reactions with sulfo-cyanine5 (Cy5) azide to generate far-red fluorescent probes (Figure 3B): aztreonam-Cy5 (Azt-Cy5), cephalexin-Cy5 (CephX-Cy5), and meropenem-Cy5 (Mero-Cy5). The fluorescent probes were made in small quantities (e.g., low microgram scale) and used without further purification.

We used Bocillin-FL for the initial evaluation of enzyme targets in *Mtb* lysates (Figure 4). Prior work used this probe to detect PBPs—including PonA2—in *Mycobacterium smegmatis* (*Msmeg*), a rapidly growing mycobacterial species.^{20,52} We analyzed lysates prepared from mid log phase cultures of *Mtb* (mc²6020), a *lysA panCD* auxotroph of *Mtb* H37Rv.⁵³ Bocillin-FL-labeled lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and imaged on a fluorescence scanner to detect green fluorescence from the target-bound probe (Figure 4). Labeled proteins were nearly all over 50 kDa, despite 10 of the targets in Table 1 being of a lower molecular weight. Labeling was reduced by pretreatment with penicillin G and other β -lactam antibiotics (see Supporting Information; Figure S1). This competition experiment demonstrated that Bocillin-FL labeling was specific for proteins that bind to β -lactams. The fluorescent band intensity corresponded with activity not relative protein abundance (see Figure S1B). We observed three autofluorescent bands in the green channel. Additionally, there were three major drawbacks to using Bocillin-FL: (1) Bocillin-FL labeled less than half of the potential targets; (2) it labeled only two proteins under 50 kDa; (3) penicillins are not inhibitors of LDTs.

We overcame those drawbacks by using our far-red fluorescent ABPs: Azt-Cy5, CephX-Cy5, and Mero-Cy5. *Mtb* lysates were probe-treated, resolved by SDS-PAGE, and imaged to detect Cy5-labeled targets (Figure 4). All of these probes out-performed Bocillin-FL by revealing more fluorescent bands (i.e., enzymes inhibited by β -lactams). They also labeled multiple proteins with an apparent molecular weight of less than 50 kDa. This is further evidence that Bocillin-FL is a poor substrate for targets below 50 kDa because the lysis, treatment, and gel conditions were similar among the four fluorescent probes. This finding is consistent also with prior work that found that penams do not covalently inhibit the active site of LDTs,¹¹ which have a molecular weight range of 26.9–47.9 kDa.

We performed competition experiments with the parent antibiotic, and labeling was reduced under these conditions. Some bands remained unchanged in the cephalexin-treated lysates, suggesting that CephX-Cy5 may have some non-specific interactions with proteins. However, pretreatment with aztreonam or meropenem significantly reduced labeling by the corresponding probe. This indicates that the labeling with Azt-Cy5 and Mero-Cy5 was specific for proteins that bind β -lactams. Notably, these new probes each revealed distinct target selectivity plus bands that were undetected by Bocillin-FL. All four probes detected BlaC activity at 30 kDa (see next section).

Identification of Protein Targets of Mero-Cy5.

We initiated the process of identifying proteins that were labeled by the carbapenem probe Mero-Cy5. In these studies, we used lysates from actively replicating cultures and dormant cultures of *Mtb*. We induced dormancy by incubation under hypoxic conditions, as described.^{5,54} *Mtb* lysates were fractionated by centrifugation (into pellet and supernatant), labeled with Mero-Cy5, and resolved by SDS-PAGE. The pellet was enriched in proteins associated with the cell wall and cell membrane (Figure S2).⁵⁵ Fluorescent bands were excised and submitted for identification by liquid chromatography tandem mass spectrometry (LC-MS/MS).

We focused our analysis on proteins with putative PBP, LDT, or β -lactamase activity (see Table S1). Of the 43 proteins in Table S1, 32 were identified in our MS analysis (Table S2). Experimental details and the full data set are provided in the Supporting Information (SI), including annotated gels from which we excised bands (Figures S2–S6). A summary of the results from these studies is provided in Figure 5. Specifically, we identified DDTs (PonA1, PonA2, PBPA), LDTs (Ldt_{M12}, Ldt_{M13}, Ldt_{M15}), carboxypeptidases (DacB1, DacB2, PBP4), and the β -lactamase BlaC. Most of these enzyme hits were identified in more than one sample.

We further investigated one of the identified enzymes: BlaC. BlaC's activity most likely contributes much of the signal at 30 kDa. In normoxic samples, we identified 18 unique peptides from BlaC with 69.7% sequence coverage (see Figure S3). We validated our assignment by comparing lysates from *Mtb* (mc²6020) with *Mycobacterium marinum*, a close genetic relative, and with *Msmeg* (mc²155), a species that lacks a BlaC ortholog. We observed a clavulanate-sensitive band at 30 kDa for lysates from *Mtb* and *M. marinum* but not *Msmeg* (Figure S4). The major β -lactamase in *Msmeg*, BlaS (31 kDa), is not a target of clavulanate.⁵⁶ This comparative analysis provides compelling support for our assignment of the fluorescent band at 30 kDa to BlaC activity.

Interestingly, we observed a different banding pattern near 30 kDa for samples analyzed from normoxic versus hypoxic cultures. We identified peptides from BlaC and carboxypeptidase DacB2; other β -lactamases were identified in considerably lower abundance in these excised bands. This made the source of the signal ambiguous because it could be from any combination of BlaC, DacB2, or the putative β -lactamases. While it has been shown that BlaC represents the majority of β -lactamase activity under regular growth conditions,³⁸ similar studies have not been conducted with non-replicating bacteria. Further studies will be needed to learn more about the source of this up-regulated activity.

Next, we analyzed the middle region of the gel, which contained three fluorescent bands between 40 and 50 kDa (Figure S5). The fluorescent band directly above 50 kDa was identified as PBPA (51.6 kDa), on the basis of the MS data (77.4% sequence coverage, 28 peptide hits). The band just below 50 kDa contained peptides from PBP4 (46.8 kDa) and Ldt_{M15} (47.9 kDa). The analysis of the band just above 40 kDa resulted in masses corresponding to 14 proteins with relevant Pfam notation, including eight putative β -lactamases, four PBPs (PBP4, DacB1, PBPA, and PonA2) and two LDTs (Ldt_{M15} and Ldt_{M12}). By molecular weight, DacB1 (41.7 kDa) and Ldt_{M12} (43.4 kDa) are the most likely

to be present in this region. Ldt_{M12} was identified from three separate samples, supporting its contribution to the fluorescent band near 40 kDa.

We obtained peptide hits from both PonA1 and PonA2 in the bands excised near 80 kDa. We believe that the signal from the top and bottom of the four bands is from PonA1 because only that protein was identified in bands excised from hypoxic samples (Figure S6). The appearance of PonA1 in two locations is unsurprising because it is regulated by phosphorylation,⁵² which can influence protein migration by SDS-PAGE. We verified the presence of phosphorylated proteins in those bands by staining the gel with Molecular Probe's Pro-Q Diamond stain (Figure S7). We believe that the two middle bands contain predominantly PonA2, with the lower band resulting from cleavage of a 3 kDa signal sequence, which was only identified in the upper of those two bands.

These studies enabled us to identify a variety of targets in gel-resolved lysates, including DDTs (PonA1, PonA2, PBPA), carboxypeptidases (DacB1, DacB2, PBP4), LDTs (Ldt_{M12}, Ldt_{M13}, Ldt_{M15}), and the β -lactamase BlaC. Additionally, we identified 22 other potentially relevant proteins (see Table S2), which are worth investigating in future work. Our findings are in stark contrast to a 2020 report by Lopez Quezada and coworkers.⁵⁷ In their work, non-replicating *Mtb* was treated with a cephalosporin-based ABP. Labeled protein targets were enriched and identified by MS-based proteomics. No PBPs or LDTs were identified, supporting their claim that those compounds only target enzymes that are not transpeptidases. Clearly there is more to learn about how different classes of β -lactam drugs exert their effect in *Mtb*.

Patterns of Enzyme Activity from Hypoxic and Normoxic *Mtb*.

Our MS analysis suggested that the activity patterns of PBPs and LDTs change in response to hypoxia. This fits prior observations that *Mtb* increases LDT-mediated (3 → 3) cross-links in dormancy.^{14,15,23} To better understand these differences, we analyzed ABP-labeled lysates resolved by SDS-PAGE (Figure 6). In these studies, we normalized by total protein to enable a direct comparison between band intensities observed under different culture conditions (i.e., normoxic versus hypoxic). We found that many targets were present in both metabolic states. For example, PonA1, PonA2, PBPA, Ldt_{M12}, Ldt_{M13}, and Ldt_{M15} maintained activity in dormancy. Other targets, particularly between 40 and 80 kDa, were less active in dormancy, and at least three bands were absent. Unfortunately, we were unable to determine which enzymes lost activity in dormancy, highlighting a challenge of identifying low abundance proteins from gel-excised bands.

The cephalixin probe indicated some up-regulation of faint bands in the 80 kDa region of the gel. These changes were also visible with Azt-Cy5, Mero-Cy5, and Bocillin-FL (Figure S8). It would be interesting to know if these changes are a result of post-translational modifications, such as glycosylation or phosphorylation.⁵² Bocillin-FL offered little insight on enzyme targets below 50 kDa, as expected.

As noted in the previous section, we observed up-regulated enzyme activity near 30 kDa from hypoxic lysates. In particular, the ~28 kDa band was only distinct in hypoxic samples. This region was also notable in lysates treated with Azt-Cy5, which showed similar up-

regulation of bands from 25 to 30 kDa under hypoxia (Figure S8). Our proteomics results indicate that DacB2 migrates in this region of the gel. This suggests that DacB2 could have enhanced activity under hypoxic conditions, a notable result considering that the related carboxypeptidase DacB1 is transcriptionally up-regulated in dormancy.¹⁶ To summarize, our in-gel analysis provides evidence that the PBPs and LDTs are dynamically regulated in response to hypoxia.

Drug Susceptibility of PBPs and LDTs in *Mtb*.

There is evidence that β -lactams are therapeutically relevant drugs for TB patients.^{27,51,58} For example, there have been occasional reports of successfully treating TB patients with the coadministration of β -lactams with clavulanate, a BlaC inhibitor.^{51,59–65} A 1998 report described using amoxicillin/clavulanate to cure multidrug-resistant TB.⁶⁴ Drug-resistant TB has also been treated with meropenem/clavulanate.^{59–61,63,65} That treatment helped to cure extensively drug-resistant TB patients (83% cure rate).⁶³ This drug combination was also effective against dormant *Mtb*.^{66,67}

Inspired by those findings, we set out to investigate the targets of β -lactam drugs in *Mtb* using ABPs. We tested cephalexin, ceftriaxone, aztreonam, penicillin G, ampicillin, carbenicillin, meropenem, faropenem, and clavulanate. All of these drugs, except faropenem, are prescribed for treating bacterial infections in the United States. For these studies, we analyzed ABP-labeled lysates alongside lysates pretreated with a selection of β -lactam antibiotics as inhibitors (Figure 6). Again, we studied lysates from dormant (hypoxic) and actively replicating cultures. After drug treatment, lysates were labeled with Mero-Cy5. As an additional control, we exposed lysates to a sulfo-Cy5 conjugated to 5-hexynoic acid instead of a β -lactam (Lane 12). The absence of labeling with this compound indicated that the β -lactam, not the Cy5 fluorophore, dictated target-binding specificity.

We were most interested in identifying enzymes that are targets of the carbapenems (i.e., meropenem and faropenem) because these drugs inhibit LDTs²⁷ and undergo slower hydrolysis by BlaC compared with other β -lactam classes.³¹ As expected, pretreatment with meropenem (Lane 8), the closest structural analogue to Mero-Cy5, eliminated most of the fluorescent labeling. We observed that meropenem completely inhibited a band that corresponds to Ldt_{Mt5}, which is consistent with the finding from Lamichhane et al. that meropenem acylates the Ldt_{Mt5} active site.⁴⁷ Similar results were found with faropenem (Lane 9).

Ceftriaxone (Lane 3), a clinically approved cephem, was more effective than cephalexin (Lane 2) at blocking Mero-Cy5 binding, although neither antibiotic was an effective inhibitor of BlaC, Ldt_{Mt5}, or Ldt_{Mt3}. The penams (penicillin G and ampicillin) and carbenicillin (Lane 7) were less effective at inhibiting targets than other drugs but reduced the Mero-Cy5 labeling of PBPA. BlaC, which retained some activity in dormancy, was inhibited by several drugs (Lanes 7–10), including clavulanate (Lane 10) and meropenem (Lane 8).

Antibiotic susceptibility was also analyzed using Azt-Cy5 and CephX-Cy5, and we observed similar inhibition profiles (Figure S9). Overall, our findings indicate that PBP and LDT activities can be reduced or eliminated *in vitro* using clinically approved β -lactams.

CONCLUSION

In conclusion, we used fluorescent β -lactam probes to identify active PBPs, LDTs, and β -lactamases in *Mtb*. In a direct comparison with Bocillin-FL, we found that Azt-Cy5, CephX-Cy5, and Mero-Cy5 enabled more β -lactam targets to be detected in protein gel-resolved lysates. Although we collected data with Cy5-modified probes, we reported the synthesis of the alkyne derivatives because they enable facile modification with an assortment of reporters. For example, a biotinylated probe would enable the enrichment of rare or low-abundance β -lactam targets before proteomic analysis.⁶⁸

We used Mero-Cy5, a new probe, to identify 32 proteins that bind β -lactams, including transpeptidases (PonA1, PonA2, PBPA, Ldt_{Mt2}, Ldt_{Mt3}, Ldt_{Mt5}), carboxypeptidases (DacB1, DacB2, PBP4), BlaC, and numerous putative β -lactamases (see Table S2). We did not identify Ldt_{Mt1} or Ldt_{Mt4}, although there is structural evidence that both LDTs bind carbapenems.^{32,46,69} Future studies might use purified proteins or genetic knockout strains to more definitively assign activities identified here.

Mero-Cy5 was used to investigate enzyme regulation in dormant and actively replicating samples. We found numerous changes in fluorescent banding patterns and intensity between the two metabolic states. These findings provide direct evidence for the first time that the PBP and LDT activities are different between dormant and actively replicating cultures. For example, we found that some bands (25–30 kDa) were up-regulated under hypoxia. A priority for future work is to identify the exact source of that activity because it is unclear if those bands are attributable to changes in the activity of BlaC, DacB2, or an uncharacterized protein. We are in the early stages of confirming these findings under other conditions that induce dormancy (e.g., carbon starvation³ or a multistress model⁵⁷). Also, it would be interesting to study in more detail how various post-translational modifications alter PBP and LDT activities during different stages of infection.

Lastly, we treated lysates with a set of β -lactam drugs to identify their protein targets. We focused on a set of antibiotics in clinical use today and found that meropenem and faropenem were both good inhibitors of BlaC, PBPs, and LDTs. Overall, we demonstrated that Mero-Cy5 is a useful probe for target identification, the analysis of enzyme regulation, and determining enzyme inhibition by various β -lactam drugs.

It is worth noting that the far-red fluorescent probes described herein were developed with fluorescence imaging in mind. Prior studies have demonstrated that peptidoglycan biosynthesis occurs at both the poles and sidewall of mycobacteria.^{25,26,70} Mycobacteria have been labeled using various small molecule probes compatible with fluorescence microscopy, including fluorescent peptides,⁷⁰ trehalose analogues,²⁵ and fluorescent D-amino acids.^{25,26} In future work, we intend to examine LDT and PBP activity in the *Mtb* cell wall using fluorescent β -lactams, including Mero-Cy5. Alone or in combination with

existing probes, Mero-Cy5 could be used to highlight the portions of the cell wall that have the requisite machinery for cross-linking peptidoglycan. These small molecule probes are compatible with live cell labeling and would enable new investigations into how PBPs or LDTs are regulated during cell division, pathogenesis, or dormancy.

METHODS

Synthesis of Activity-Based Probes.

Bocillin FL was obtained from ThermoFisher Scientific. Azt-alkyne, CephX-alkyne, and Mero-alkyne were synthesized from the parent β -lactam, as described in the Supporting Information. The red-fluorescent probes were generated *in vitro* via a CuAAC reaction (1 h, room temperature) of the alkyne (100 μ M) with sulfo-Cyanine5-azide (100 μ M), 1.25 mM THPTA/0.25 mM CuSO₄, and sodium ascorbate (15 mM) in HEPES buffer (pH 7.3); the fluorescent probes were used without purification.

Mtb Culture Conditions.

Mtb mc²6020 was grown in Middlebrook 7H9-OADC medium supplemented with lysine, pantothenate, and casamino acids. For normoxic cultures, the flasks were maintained at 37 °C with 100 rpm shaking under atmospheric conditions. For hypoxic cultures, mid log phase cultures were diluted to OD₆₀₀ 0.4 and grown as standing cultures under hypoxic conditions (1% O₂ and 5% CO₂) at 37 °C until a control flask containing methylene blue was decolorized.⁷¹

Preparation of Lysates.

Mtb grown under normoxic conditions was harvested at mid log phase (OD₆₀₀ = 0.9–1.2). *Mtb* grown under hypoxic conditions was harvested at fixed time-points (OD₆₀₀ = 0.4–0.8). All cells were collected by centrifugation (10 min, 4000g, 4 °C) and washed twice with PBS containing 0.05% Tween 80. Pellets were resuspended in detergent-free lysis buffer [50 mM Tris (pH 7.5 at 4 °C), 50 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂], lysed by mechanical disruption, and pelleted by centrifugation (10 min, 4000g, 4 °C). The supernatant was transferred to a separate tube. The beads and cell debris were resuspended in an equivalent volume of double detergent containing lysis buffer [50 mM Tris (pH 7.5 at 4 °C), 50 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.4% triton X-100], and the mechanical disruption and centrifugation steps were repeated. The combined supernatants were pelleted by centrifugation (10 min, 4000g, 4 °C) to remove insoluble debris and sterilized by filtration (0.2 μ m PES membrane).

Lysate Labeling and Gel Imaging.

Lysates were diluted with detergent containing buffer [50 mM Tris (pH 7.5 at 4 °C), 50 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.2% triton X-100] to normalize the total protein concentration. For samples pretreated with antibiotics, the antibiotic was added for 15 min prior to probe labeling. Lysates were labeled with the Cy5-modified probe (5 μ M) for 1 h at room temperature. Lysates were resolved by SDS-PAGE, washed, fixed, and imaged on a Typhoon multimode imager.

Band Excision and LC–MS/MS Proteomics.

All bands were excised from an SDS-PAGE gel on the basis of the fluorescent image and stored frozen. Gel slices were processed to remove SDS, reduced, and methylated. Proteins were trypsin-digested, and the resulting peptides were concentrated. LC–MS/MS-based proteomics was performed by the OHSU Proteomics Shared Resource Facility. Peptides were considered positively identified if their probability of identification was >99% with a false-discovery rate (FDR) of 0.67%. Proteins were considered positively identified if two or more unique peptides were detected (probability of identification >95%; FDR 0.0%).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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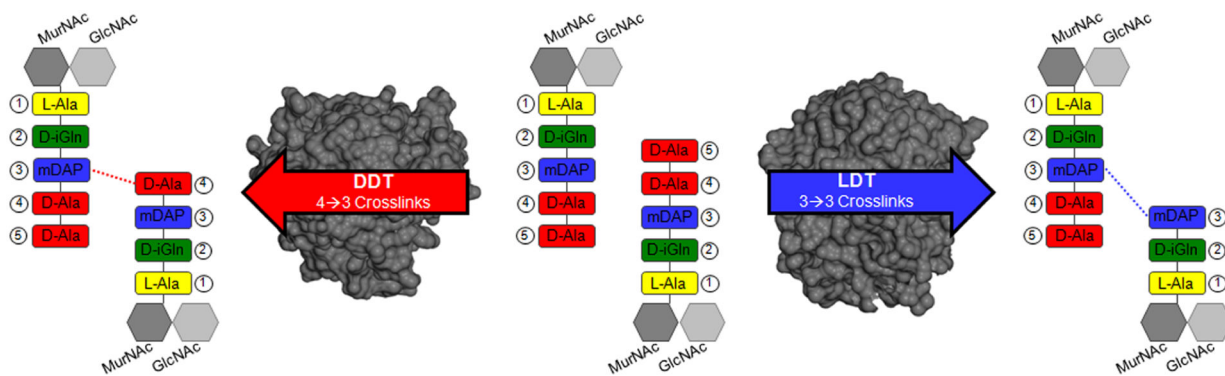


Figure 1.

PBPs and LDTs catalyze transpeptidase reactions between different amino acid substrates. A subset of PBPs is called D,D -transpeptidases (DDTs), and they cross-link a position 4 D-Ala to a neighboring mDAP residue. *Mtb* also uses L,D -transpeptidases (LDTs), which form 3 \rightarrow 3 cross-links (mDAP to mDAP). The DDT protein depicted in dark gray (left) is PonA1 (PDB ID: 5CXW) and the LDT structure (right) is Ldt_{Mt2} (PDB ID: 5K69). (Dark gray hexagon) MurNAc, *N*-acetylmuramic acid; (Light gray hexagon) GlcNAc, *N*-acetylglucosamine; L-Ala, L-alanine; D-iGln, D-isoglutamine; mDAP, meso-diaminopimelic acid; D-Ala, D-alanine.

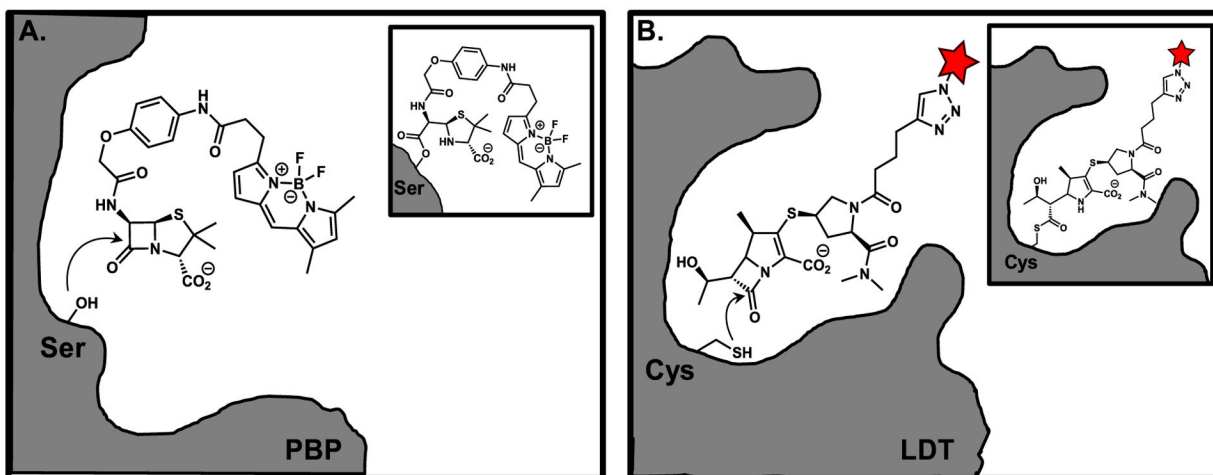
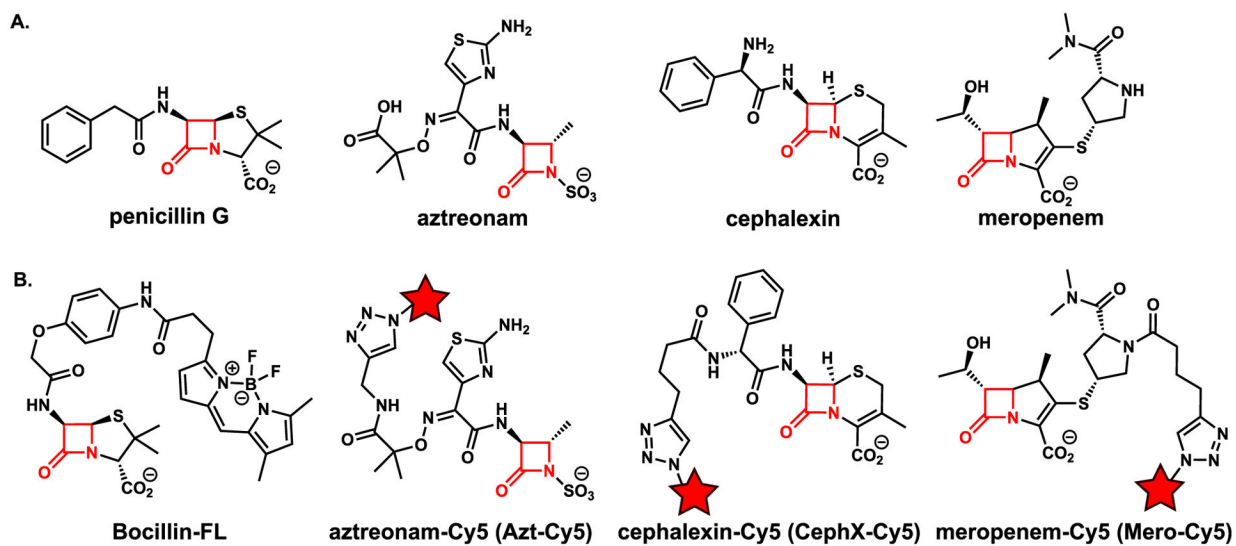


Figure 2.

Active-site labeling of PBPs and LDTs with β -lactam probes. (A) In PBPs, an active-site serine binds to a reactive β -lactam probe, as shown for Bocillin-FL. (B) In contrast, the LDT's active-site cysteine binds to carbapenems (e.g., meropenem-Cy5). Insets: Active-site covalently bound to the β -lactam probe.

**Figure 3.**

Structures of (A) β -lactam antibiotics and (B) activity-based probes used in the current work. The Cy5-conjugated derivatives were obtained by reacting the alkyne probe with sulfo-cyanine5 (Cy5) azide to produce Azt-Cy5, CephX-Cy5, and Mero-Cy5. The red star denotes the Cy5 fluorophore, and the β -lactam ring is highlighted in red.

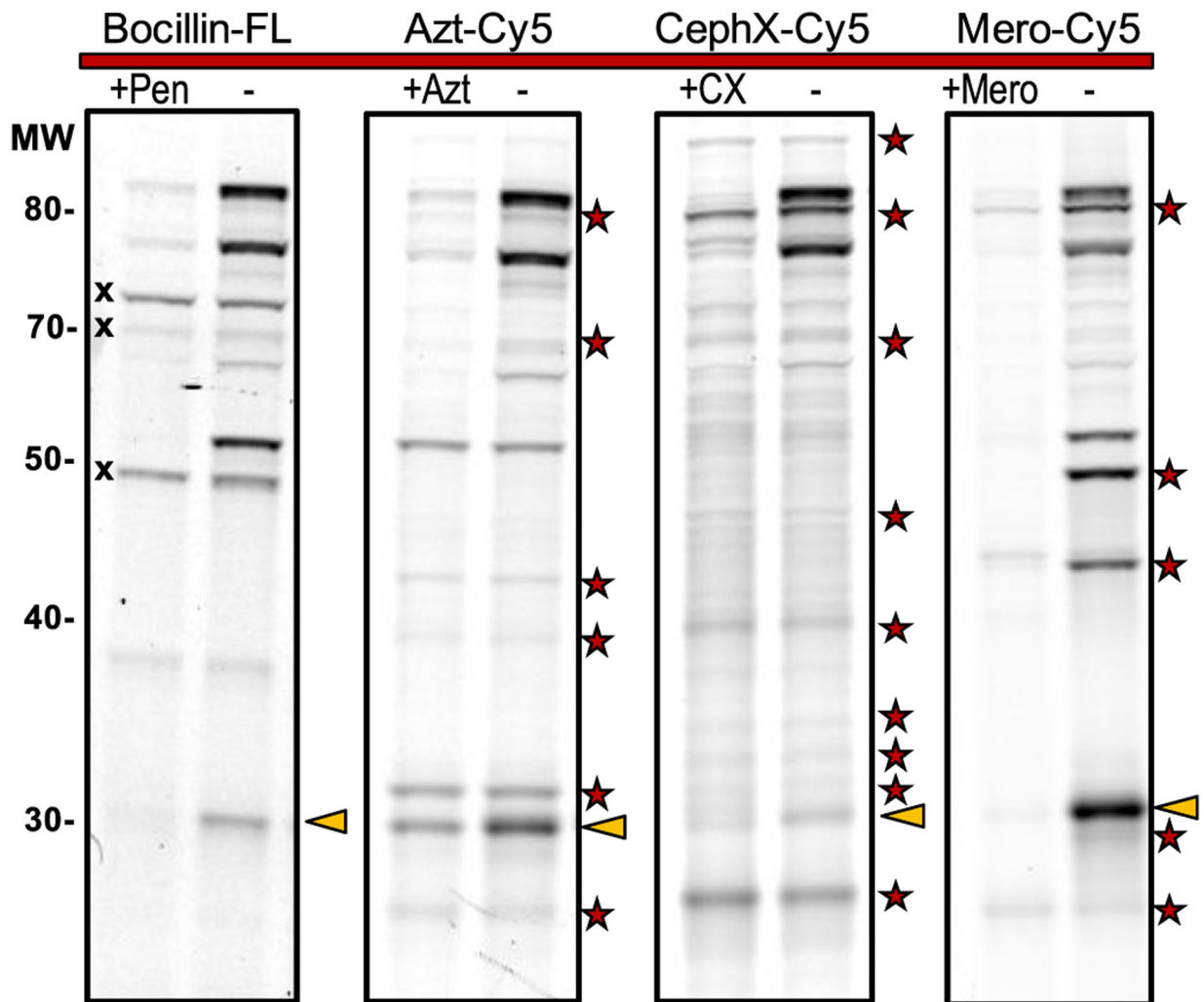


Figure 4.

β -Lactam probes covalently bind to *Mtb* proteins, enabling fluorescent detection. Lysates were treated with a probe, resolved by SDS-PAGE, and imaged. Three autofluorescent bands (X) were observed with Bocillin-FL but not with Cy5 probes. Labeling was reduced by pretreatment with the parent antibiotic (left lanes). Pen, penicillin G; Azt, aztreonam; CX, cephalexin; Mero, meropenem. Red star: Targets bound and detected by Azt-Cy5, CephX-Cy5, or Mero-Cy5 (but not Bocillin-FL). BlaC migrates at 30 kDa and is denoted by gold triangles.

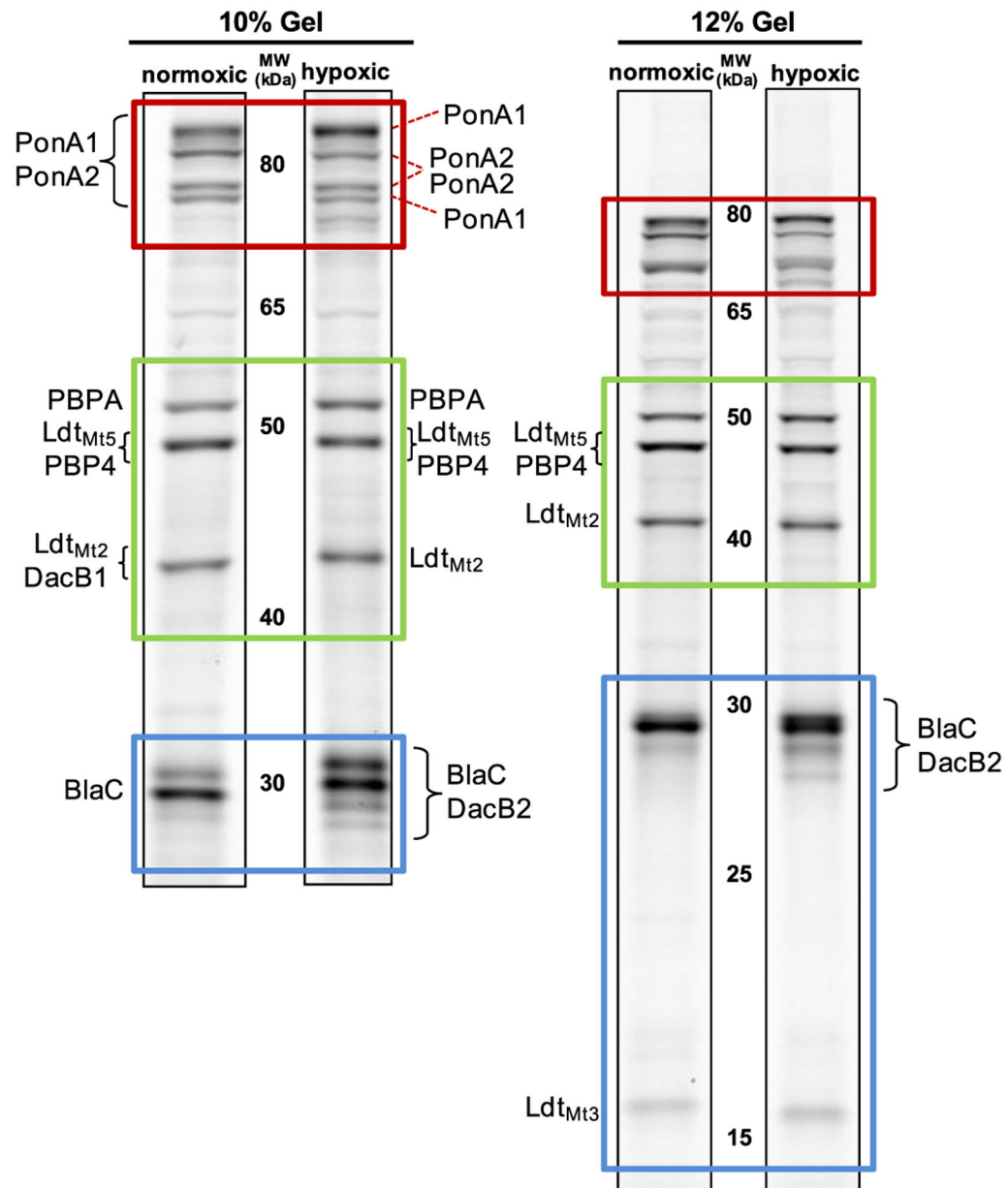


Figure 5. Analysis of *Mtb* lysates for MS-based identification of proteins labeled with Mero-Cy5. Lysates from normoxic and hypoxic cultures were resolved on 10% (left) or 12% (right) SDS-PAGE protein gels. Based on the data provided in the SI, the gels have been annotated to indicate the most likely PBP or LDT source of fluorescent bands. To simplify comparisons, three regions of the gels are boxed: High MW (~80 kDa; red); Middle MW (40–50 kDa; green); Low MW (15–30 kDa; blue). Figures S3, S5, and S6 include more information for each excised band, including protein identities, molecular weight, % coverage, and the number of peptides found per protein.

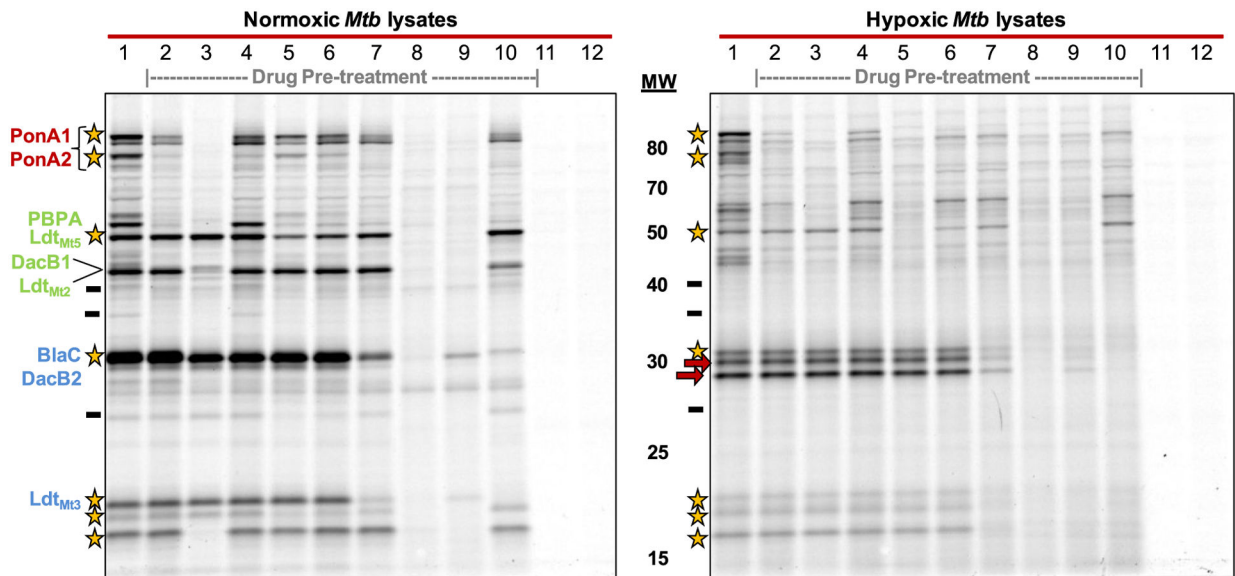


Figure 6.

Mero-Cy5 reveals relative enzyme activities and drug targets in lysates obtained from normoxic and hypoxic *Mtb* cultures. Lane 1: no drug pretreatment. Lanes 2–10: drug pretreatment. 2, cephalexin; 3, ceftriaxone; 4, aztreonam; 5, penicillin G; 6, ampicillin; 7, carbenicillin; 8, meropenem; 9, faropenem; 10, clavulanate. Lane 11: lysates *not* treated with Mero-Cy5. Lane 12: lysates treated with a Cy5-(triazole)-butanoic acid. Putative protein identities were obtained by MS analysis. Legend: Gold stars denote enzymes that retain activity under hypoxic conditions. Red arrows indicate bands with enhanced activity in hypoxia. Black dash-marks indicate bands that were absent in hypoxia but present in normoxia.

Table 1.

LDTs and PBPs in *M. tuberculosis* (*Mtb*)

	name	RV #	enzyme type	MW (kDa)
L,D-transpeptidases (LDTs)	Ldt _{Mt1}	Rv0116c	LDT	26.9
	Ldt _{Mt2}	Rv2518c	LDT	43.4
	Ldt _{Mt3}	Rv0433	LDT	28.7
	Ldt _{Mt4}	Rv0192	LDT	38.9
	Ldt _{Mt5}	Rv0483	LDT	47.9
penicillin-binding proteins (PBPs)	PonA1	Rv0050	transglycosylase and DDT	71.1
	PonA2	Rv3682	transglycosylase and DDT	84.6
	PBPA	Rv0016c	DDT	51.6
	PBPB	Rv2163c	DDT	72.5
	PBP-lipo	Rv2864	DDT	63.0
	PBP4	Rv3627c	carboxypeptidase	46.8
	DacB1	Rv3330	carboxypeptidase	41.7
	DacB2	Rv2911	carboxypeptidase	29.7
	BlaC	Rv2068c	β -lactamase	32.6
		Rv0907	β -lactamase	56.7
		Rv1367	β -lactamase	41.3