

RESEARCH ARTICLE

Ldt_{Mav2}, a nonclassical transpeptidase and susceptibility of *Mycobacterium avium* to carbapenemsRohini Mattoo¹, Evan P Lloyd², Amit Kaushik¹, Pankaj Kumar¹, Julie L Brunelle³, Craig A Townsend^{2,4} & Gyanu Lamichhane^{*1,4}

Aim: *Mycobacterium avium* infections, especially in immune-compromised individuals, present a significant challenge as therapeutic options are limited. In this study, we investigated if *M. avium* genome encodes nonclassical transpeptidases and if newer carbapenems are effective against this mycobacteria. **Materials & methods:** Biochemical and microbiological approaches were used to identify and characterize a nonclassical transpeptidase, namely L,D-transpeptidase, in *M. avium*. **Results & conclusion:** We describe the biochemical and physiological attributes of a L,D-transpeptidase in *M. avium*, Ldt_{Mav2}. Suggestive of a constitutive requirement, levels of Ldt_{Mav2}, a L,D-transpeptidase in *M. avium*, remain constant during exponential and stationary phases of growth. Among β -lactam antibacterials, only a subset of carbapenems inhibit Ldt_{Mav2} and tebipenem, a new oral carbapenem, inhibits growth of *M. avium*.

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A recent report suggests that global incidence of *Mycobacterium avium* infections is significant and underestimated [1]. In immune-compromised hosts, for instance, patients infected with HIV, *M. avium* can cause chronic-disseminated disease with significant morbidity and mortality [2]. *M. avium* infections are difficult to treat as this pathogen is naturally resistant to most antibacterials available today. Therefore, new insights into vulnerabilities of this pathogen, that may be exploited to develop effective therapies, can have an immediate and a lasting impact.

Mycobacteria possess a large cell wall that accounts for approximately 40% of cell's dry mass [3]. As a major component of the cell wall, the peptidoglycan serves as an anchor to vital cell wall molecules via covalent linkage to arabinogalactan layer to which mycolic acids are associated [4]. Until recently, it was widely accepted that D,D-transpeptidases (commonly known as penicillin-binding proteins) catalyzed the final step of peptidoglycan biosynthesis by linking the fourth amino acid of one stem peptide to the third amino acid of another thereby generating a 4 \rightarrow 3 linked 3D-exoskeleton. However, the peptidoglycan of *Mycobacterium tuberculosis*, *Mycobacterium abscessus* and *Mycobacterium smegmatis* are predominantly cross-linked with 3 \rightarrow 3 linkages generated by non-classical transpeptidases, namely L,D-transpeptidases [5–9]. Unlike D,D-transpeptidases that use serine to catalyze transpeptidation reaction, the catalytic residue in L,D-transpeptidases is cysteine which when mutated to serine completely abrogates its

KEYWORDS

- carbapenem
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activity [10]. The predominance of 3→3 cross-linked peptidoglycan in *M. tuberculosis* is an important reason why β-lactam sub-classes, penicillins and cephalosporins are ineffective against this pathogen [6,11]. Penicillins and cephalosporins are also not considered for treatment of *M. avium* infection as their minimum inhibitory concentration (MIC₉₀) against this pathogen are approximately 40–160 μg/ml [12] and therefore therapeutically irrelevant. In addition to the native β-lactamase present in *M. avium* [13], the presence of L,D-transpeptidases could potentially contribute toward natural resistance to penicillins and cephalosporins as this class of β-lactams are not known to be effective against L,D-transpeptidases [10,11]. An *M. tuberculosis* strain lacking the dominant L,D-transpeptidase, Ldt_{Mt2}, manifested altered cell and colony morphologies, loss of virulence and enhanced sensitivity to β-lactams [6,14]. Here, we began with genomic approaches to identify Ldt_{Mt2} orthologs of *M. tuberculosis* L,D-transpeptidases in *M. avium*, and focused on an ortholog of Ldt_{Mt2}, hereafter referred to as Ldt_{Mav2}. We used biochemical, biophysical, genetic and microbiological approaches to characterize this protein. We explored localization, abundance and relevance of Ldt_{Mav2} to physiologically relevant stresses and characterized its interactions with β-lactams. Although generally not considered for treatment of *M. avium* infections, data from our study merit further investigation of the therapeutic utility of carbapenems against this pathogen. In particular, our finding that a new carbapenem that is orally bioavailable, namely tebipenem, has potent *in vitro* activity can have direct impact on the treatment of *M. avium* infections that are resistant to existing drugs.

Materials & methods

• Bacterial strains & growth conditions

M. avium 104, a clinical isolate [15], was used in this study and was grown in Middlebrook 7H9 broth (Becton and Dickinson, Sparks, MD, USA) supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC), 0.2% glycerol, 0.05% Tween-80 and 50 μg/ml cycloheximide (7H9 complete medium) at 37°C with constant shaking. To assess response to stress, *M. avium* was grown in 7H9 complete medium to exponential phase ($A_{600\text{ nm}} = 0.6$), centrifuged, washed once with 7H9 broth and

resuspended in 7H9 complete medium alone (control), H₂O or 7H9 medium supplemented with the following: 0.05% sodium dodecyl sulfate (SDS) or 2 μg/ml crystal violet or 1 μg/ml tebipenem. *M. avium* was also incubated in Sauton's medium alone and Sauton's medium supplemented with 40 μM ferric ammonium sulfate. *Escherichia coli* strains, DH5α and BL21(DE3), were grown in Luria-Bertani (LB) broth or LB agar with appropriate selection drug.

• Sequence analysis of Ldt_{Mav2}

Sequence of Ldt_{Mav2} was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) *M. avium* 104 database [16]. Sequence alignment to compare primary structure of Ldt_{Mav2} with its orthologs in other mycobacteria and to infer residues potentially involved in catalysis was undertaken using Multiple Alignment using Fast Fourier Transform (MAFFT) [17]. Furthermore, Clustal X was used to color classify each amino acid. The cladogram construction of the sequences was carried out using MEGA 6.06 [18].

• Cloning, overexpression & purification

The N-terminal 24 residues of Ldt_{Mav2} were excluded to avoid the likely transmembrane anchor predicted by TMHMM [19] that could hinder purification of the protein. Primers attgccatagggtggggcagtcgcctgcccgc and caatactcgagtcaggtgttggcgttggcccgc were used to amplify the region-encoding residues 25–403 and cloned into pET28a+TEV to generate an N-terminal H₆ tagged Ldt_{Mav2}. *E. coli* BL21δε3 was used to overexpress Ldt_{Mav2}, treated with TEV protease to cleave the H₆ tag while simultaneously dialyzed to remove imidazole. Gel filtration was carried out using Superdex-200 10/300 GL (GE Healthcare, IL, USA) to obtain monomeric Ldt_{Mav2}.

• Biochemical characterization

The optimum pH for Ldt_{Mav2} was determined by assessing absorbance of opening of the β-lactam ring of nitrocefin at 490 nm, molar extinction coefficient of 20,500 M⁻¹cm⁻¹ as described [20]. Reaction mixtures contained 10 μM Ldt_{Mav2}, 100 μM nitrocefin, 100 mM-modified buffer containing 2-(N-Morpholino)ethanesulfonic acid (MES), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and diethanolamine (1:1:1) at pH ranging from 4 to 10 [21]

and 0.1 mM TCEP at 25°C in a total volume of 100 µl. To study Ldt_{Mav2} reaction kinetics, nitrocefin was used as a substrate at varying concentrations at 25°C. For each reaction, a control in which the rate of nitrocefin hydrolysis in the absence of enzyme at each pH and substrate concentration was included. The K_m and V_{max} were determined using nonlinear regression analysis of which Michaelis Menton gave the best fit using Graph pad prism (Version 6; La Jolla, CA, USA).

• Inactivation by β-lactams & mass spectrometry to identify adduct

To characterize reaction between Ldt_{Mav2} and various β-lactam subclasses, enzyme–inhibitor (EI) complex was prepared by incubating Ldt_{Mav2} with 200 µM of carbapenem at 25°C for 2 h. Unreacted inhibitor was removed by size exclusion chromatography. The purified acyl-enzyme was incubated with nitrocefin and its hydrolysis was monitored at 495 nm. Nitrocefin incubated with unreacted Ldt_{Mav2} was included as a control. To identify adduct formed with Ldt_{Mav2}, each β-lactam was incubated at 4 mM concentration with 2 µM enzyme in 400 µl of 25 mM Tris, pH 8, for 5 h at room temperature. These samples were analyzed using a Waters Aquity H-Class Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC–MS) system and adducts were identified as previously described [10].

• Determination of kinetic rate constants

The λ_{max} for each β-lactam was determined spectroscopically and they are: amoxicillin, 272 nm; cephalothin, 270 nm; doripenem, 300 nm; faropenem, 306 nm; and T210, 303.5 nm. T210 is an experimental carbapenem synthesized by our group [10]. Extinction coefficients for these compounds were determined for doripenem (7500 M⁻¹ cm⁻¹), biapenem (9845.7 M⁻¹ cm⁻¹), faropenem (6980 M⁻¹ cm⁻¹), tebipenem (6652 M⁻¹ cm⁻¹) and T210 (9880 M⁻¹ cm⁻¹). A stopped-flow apparatus (SX20; Applied Biophysics, NY, USA) was used to measure fluorescence of reaction in sodium phosphate buffer, pH 7.4 at 10°C as described [22]. Fluorescence emission was recorded at 320 nm. Fluorescence data were analyzed using regression analysis using the equation $[EI^*] = [E_{total}](1 - e^{-k_{obs}t})$. $[E_{total}]$ is the total enzyme concentration, k_{obs} is the constant to be determined and t is time. The k_{obs} values

were plotted versus the concentration of the inhibitor [I] and regression analysis was performed using the equation, $k_{obs} = k_{inact}[I]/K_{app} + [I]$, where k_{inact} is a first-order constant for acylenzyme formation and K_{app} is a constant.

• Abundance & cellular localization

Abundance of Ldt_{Mav2} was studied using western blot analysis with polyclonal antibody generated against full-length Ldt_{Mav2} in rabbits (Spring Valley Laboratory, MD, USA). *M. avium* cultures grown to exponential ($A_{600nm} = 0.6$) and stationary phases of growth ($A_{600nm} \sim 2.4$) were used to extract cytosolic and membrane protein fractions, electrophoresed on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (GE Healthcare, IL, USA). Following overnight blocking, the membrane was incubated with anti-Ldt_{Mav2} polyclonal antibody (1:1000) in Tris-buffered saline (TBS) containing 0.2% bovine serum albumin (BSA) and 0.1% Tween-80 (TBS-T) for 2 h at room temperature and was washed three-times with TBS-T for 15 min and incubated with secondary anti-rabbit-horseradish peroxidase conjugate (Cell Signaling, MA, USA). The blot was washed three-times with TBS-T, and developed using chemiluminescence. Purified Ldt_{Mav2} was included as a control.

Cellular localization of Ldt_{Mav2} was studied using cell wall and cytosolic fractions of *M. avium* prepared from exponential and stationary-phase cultures using a slight modification of a validated protocol [23]. Expression levels of Ldt_{Mav2} in *M. avium* was assessed from whole-cell lysates made using *M. avium* 104 cultures grown to exponential ($A_{600nm} = 0.6$) and stationary phase ($A_{600nm} = 2.4$). The cells were centrifuged at 3000 ×g for 10 min at 4°C, the pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1 mM TCEP, 1 mM PMSF, 10% glycerol and treated with lysozyme (2 mg/ml overnight at 4°C). This sample was sonicated on an ice–ethanol mixture followed by centrifugation at 3000 ×g for 10 min to remove any unbroken cellular debris. The lysate obtained after sonication was centrifuged at 3000 ×g for 10 min to remove cellular debris and then centrifuged again at 30,000 ×g for 30 min at 4°C. The supernatant from this sample represents the cytosol and cell pellet is the cell wall fraction. The cell

lysate proteins after quantification using the modified Bradford method [24] and normalization were electrophoresed on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (GE) using a semidry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked using 5% blocking solution nonfat dry milk (Cell Signalling, MA, USA) in the presence of a buffer containing 0.2% BSA in TBS (20 mM Tris-HCl [pH 7.4], with 150 mM NaCl) overnight. Excess blocking agent was removed by washes in a buffer containing TBS (20 mM Tris-HCl [pH 7.4], with 150 mM NaCl) and 0.1% Tween-20. The membrane was then incubated with the polyclonal anti-Ldt_{Mav2} antibody (1:1000) in TBS containing 0.2% BSA and 0.1% Tween-80 for 2 h. The membrane was washed three-times with TBS containing 0.05% Tween-20 over 15 min and incubated with secondary antirabbit-horseradish peroxidase conjugate (Cell signaling) at a 1:10,000 dilution for 1 h. The blot was washed three-times with TBS-T, and was developed using chemiluminescence-developing kit (Santa Cruz Biotechnology, TX, USA), following the manufacturer's protocol. Purified MAV_1661 was used as a control.

• Protein–protein interaction

A pull-down assay was used to identify any protein(s) that interacted with Ldt_{Mav2}. *M. avium* lysate was prepared as described above from cultures harvested at exponential growth phase ($A_{600\text{ nm}} = 0.6$). The lysate was first interacted with Ni-NTA resin at 4°C for 2 h. This precleared lysate was interacted with His-tagged Ldt_{Mav2} immobilized on Ni-NTA agarose resin at 4°C for 2 h. The beads were washed with 0.5% Triton-X, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1 mM TCEP, 1 mM PMSF, 10% glycerol and 5 mM imidazole and analyzed using 10% SDS-PAGE gel. The protein band that was exclusively present in the presence of Ldt_{Mav2} was excised, digested with trypsin and subjected to mass spectrometric analysis to determine the peptide fragment masses, which were then, analyzed against the MASCOT database to identify the protein.

• Minimum inhibitory concentration & checkerboard titration assay

MIC₉₀ of agents against *M. avium* was determined using the standard broth microdilution assay [25], using 96-well plates separately

at pH 6.8 (Middlebrook 7H9 broth in the presence of 10% OADC) and pH 7.4 (cation-adjusted Mueller-Hinton broth) in accord with the Clinical and Laboratory Standard Institute (CLSI) [26] guidelines described in [27]. Standard Checkerboard Titration assay [28] was used to assess combined activity of agents. Two drugs were added to 2.5 ml of Middlebrook 7H9 broth, each starting at its MIC₉₀ and serially diluted twofold, so all possible twofold dilution combinations below respective MICs are represented. Next, 10⁵ CFU of *M. avium* were inoculated into each tube. The cultures were incubated at 37°C and evaluated for growth by visual inspection of the broth at 9 days as per CLSI guidelines. Fractional inhibitory concentration (FIC) of a drug was calculated as described [28]. FIC of a drug in a sample is computed as concentration of the drug divided by the MIC of the drug when used alone. FIC index is the sum of FIC of two drugs in a sample. FIC index was calculated for each mix of drugs that inhibited *M. avium* growth and an average FIC index was determined. Average FIC index ≤0.5 was interpreted as synergy, 0.5–2.0 as indifference and more than two as antagonism.

Results

• Ldt_{Mav2} is conserved across most mycobacteria

We used Ldt_{Mt2}, the dominant L,D-transpeptidase in *M. tuberculosis* [6], to identify its sequence ortholog, Ldt_{Mav2}, in *M. avium* 104. According to the existing annotation (accession WP_033706218.1), Ldt_{Mav2} is a 379 aa protein encoded by locus MAV_1661 [29]. Ldt_{Mav2} shares 86% sequence identity with Ldt_{Mt2} and possesses a C-terminal YkuD domain (Pfam 03734) that is characteristic of L,D-transpeptidases and two bacterial immunoglobulin-like domains (Ig) in the N-terminus (Supplementary Figure 1) [30]. While orthologs of Ldt_{Mav2} also exist in *Mycobacterium leprae* (ML0426), *M. abscessus* (MAB_1530), *M. smegmatis* (MSMEG_4745), *Mycobacterium marinum* (MMAR_3872), five additional paralogs are encoded by *M. avium* 104 genome (Figure 1A). Widespread occurrence of multiple L,D-transpeptidase orthologs across different species of mycobacteria may be indicative of their significance to the cellular physiology of this group of pathogens. As the majority of cross-linkages in the peptidoglycan of

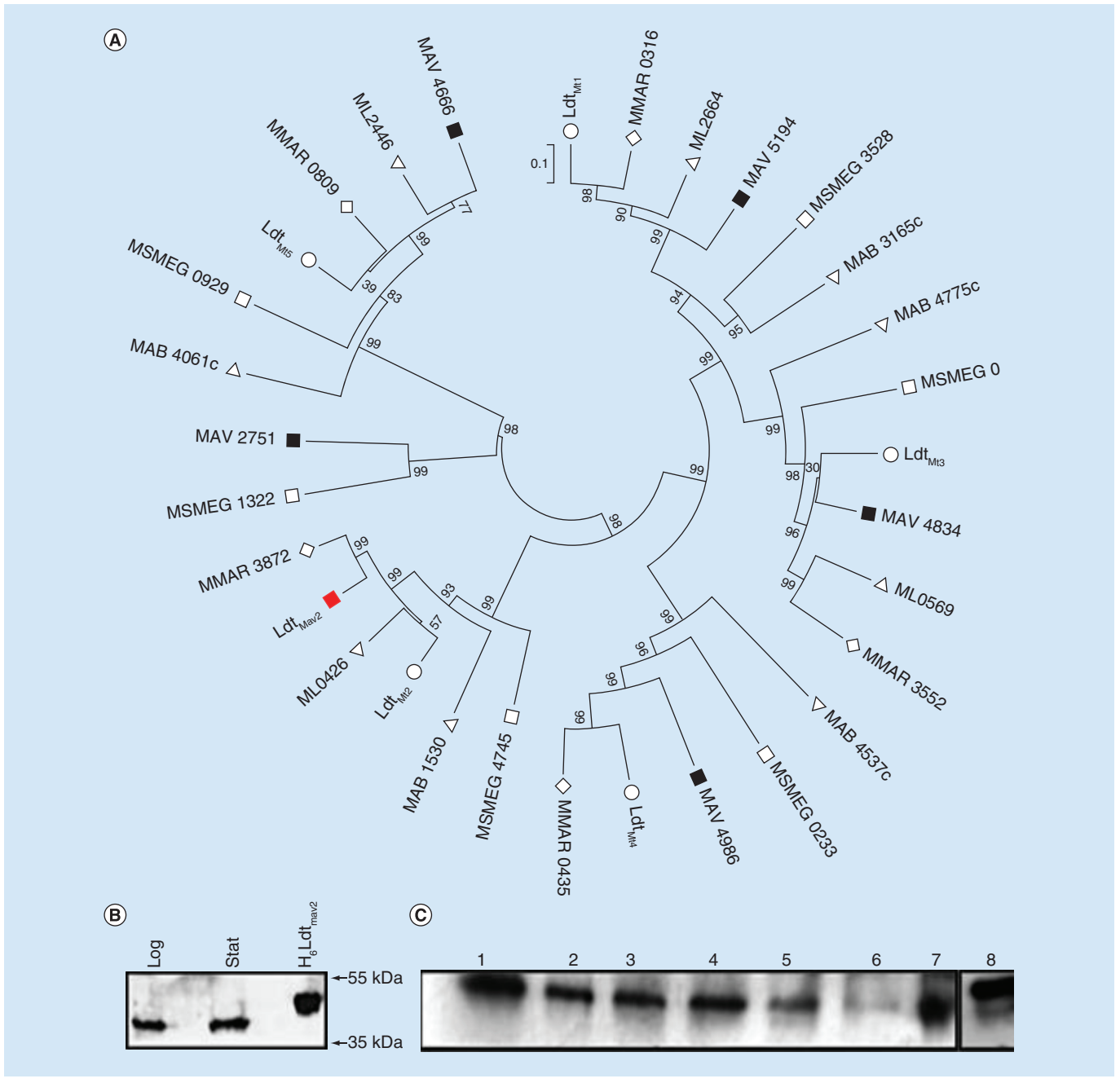


Figure 1. L,D-transpeptidases in mycobacteria and expression of Ldt_{Mav2}. (A) Cladogram of L,D-transpeptidases from various mycobacteria. Multiple alignment and phylogeny analysis of L,D-transpeptidases from *Mycobacterium tuberculosis* (O), *Mycobacterium smegmatis* (◻), *Mycobacterium abscessus* (Δ), *Mycobacterium leprae* (∇), *Mycobacterium marinum* (◊) and *Mycobacterium avium* (◻). Ldt_{Mav2} is highlighted as a red-closed square. (B) Western blot analysis of cell wall and cytosolic fractions of *M. avium* harvested at logarithmic (log) and stationary (stat) phases of growth and probed with antibody against Ldt_{Mav2}. His₆-tagged Ldt_{Mav2} is included as a control (far right). (C) Levels of Ldt_{Mav2} in the presence of 100 μM iron (lane 1), 40 μM iron (lane 2), 0.05% SDS (lane 3), 2 μg/ml crystal violet (lane 4), 1 μg/ml tebipenem (lane 5), H₂O (lane 6), Middlebrook 7H9-enriched medium (lane 7) and His₆-tagged Ldt_{Mav2} as a control (lane 8).

M. tuberculosis [5,7] and *M. abscessus* [8] are generated by L,D-transpeptidases, we hypothesize that the presence of six paralogs in *M. avium*

104 is suggestive of notable significance of this protein class to the peptidoglycan physiology in this organism.

• **Ldt_{Mav2} localizes to the cell envelope, interacts with a putative Ser/Thr kinase & responds to stress**

We used western blot analysis using an antibody raised against Ldt_{Mav2} to probe this protein in *M. avium*. We observed a single band of similar intensity in samples from both exponential and stationary growth phases but only in the cell wall fraction (Figure 1B). This protein is similar in size to Ldt_{Mav2} expressed in and isolated from *E. coli* that was included as a control. Therefore, we conclude that Ldt_{Mav2} is located in the cell wall complex that includes peptidoglycan and its abundance is similar during exponential and stationary phases of growth *in vitro* in nutrient-rich broth. In addition to the transmembrane anchor domain and the catalytic domain resembling Ldt_{Mf2} [30], Ldt_{Mav2} also possesses two Ig domains spanning residues 23–119 and 123–221. Functions, if any, of these domains are not known. We hypothesized that Ig domains in Ldt_{Mav2} may be involved in interacting with other proteins. To test this hypothesis, we performed a pull-down assay using *M. avium* whole-cell lysate and analyzed the

bound proteins using mass spectrometry. The following four proteins were present in highest abundance: Ldt_{Mav2}, trypsin, serum albumin and an uncharacterized protein of *M. avium* (accession # 497665596) with an abundance significance scores of 1593, 866, 305 and 112, respectively. Presence of Ldt_{Mav2}, trypsin and albumin (that were added during preparation of protein for mass spectrometric analysis) were expected and served as internal controls. Further analysis using BLASTp with the sequence of the bound protein identified the uncharacterized protein that bound to Ldt_{Mav2} to be a Ser/Thr kinase (Supplementary Table 1) with a homodimer interface with polypeptide-binding sites.

Given the localization of Ldt_{Mav2} in the cell wall and its likely function as a catalyst of the final step of peptidoglycan synthesis, we hypothesized that it may play a role responding to environmental stresses. We probed Ldt_{Mav2} levels in the presence of chemical stresses relevant to cell wall physiology or those that mimic stresses present during an infection. For this purpose, we exposed *M. avium* to low iron environment, osmotic shock, sublethal

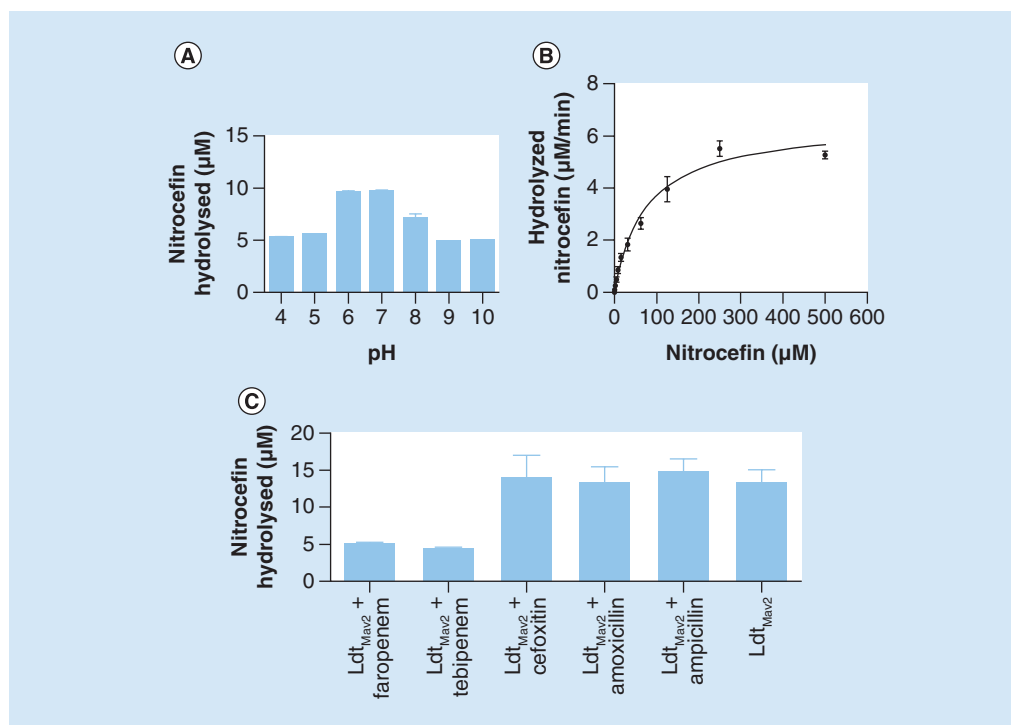


Figure 2. Biochemical characterization of Ldt_{Mav2}. (A) pH profile of nitrocefin hydrolysis by Ldt_{Mav2}. (B) Rate of nitrocefin hydrolysis by Ldt_{Mav2} at pH 6.5. (C) Assessment of nitrocefin hydrolysis by Ldt_{Mav2} following incubation with faropenem, tebipenem, cefoxitin, amoxicillin and ampicillin. The experiments were performed three-times and error bars represent standard error.

Table 1. Adducts formed between Ldt_{Mav2} and β-lactam antibacterials.

Agent	Molecular weight of adduct (Da)
Amoxicillin	No adduct
Ampicillin	No adduct
Cephalothin	+355
Cefoxitin	No adduct
Meropenem	No adduct
Imipenem	+102
Doripenem	+376, +420
Biapenem	+278
Faropenem	+86
Tebipenem	+339

Molecular weights (Da) of β-lactams: amoxicillin, 365.4; ampicillin, 349.4; cephalothin, 396.4; cefoxitin, 427.5; ceftriaxone, 554.6; meropenem, 383.5; imipenem, 299.4; doripenem, 420.1; biapenem, 350.4; faropenem, 285.3; tebipenem, 383.5.

concentrations of cell wall perturbants such as sodium dodecyl sulfate [31], crystal violet [32] and the peptidoglycan biosynthesis inhibitor tebipenem [33]. While Ldt_{Mav2} levels were similar in *M. avium* grown in the nutrient-rich Middlebrook 7H9 complete medium and Sauton's broth supplemented with 100 μM iron, a small but noticeable decrease was observed in the presence of 40 μM iron, 0.05% sodium dodecyl sulfate and 2 μg/ml crystal violet (**Figure 1C**). Exposure to 1 μg/ml tebipenem and osmotic shock resulted in very low levels of Ldt_{Mav2}.

• Ldt_{Mav2} hydrolyzes β-lactam substrate

H6-tagged Ldt_{Mav2} (25–403 aa fragment) was expressed in the heterologous host *E. coli* BL21δε3, and purified to apparent homogeneity using Ni-NTA chromatography. Further purification and assessment by gel filtration revealed that Ldt_{Mav2} is stably present in its monomeric form. Attempts to purify the YkuD domain alone (residues 253–403) were unsuccessful (data not shown), suggesting that perhaps the Ig domains are essential for stabilizing the YkuD fold. L,D-transpeptidases bind to and hydrolyze a tetrapeptide substrate during peptidoglycan biosynthesis [11]. By mimicking the native substrate, β-lactams are also bound and hydrolyzed by L,D-transpeptidases Ldt_{Mt1} [34], Ldt_{Mt2} [30] and Ldt_{Mt5} of *M. tuberculosis* [20]. Given extensive sequence similarity to Ldt_{Mt2}, we hypothesized that Ldt_{Mav2} would also bind to and catalyze hydrolysis of β-lactams. We used nitrocefin, a chromogenic β-lactam, as a substrate and observed that hydrolytic activity of Ldt_{Mav2} peaks in the physiological pH range (**Figure 2A**). Ldt_{Mav2} displayed a classical Michaelis–Menten kinetics for hydrolysis of nitrocefin with K_m and

V_{max} of 75 μM and 6.5 μM/min, respectively. Additionally the kinetic parameters with respect to hydrolysis of nitrocefin are comparable to Ldt_{Mt2} [30] and Ldt_{Mt5} [20]. The rate of nitrocefin hydrolysis was a slow reaction with a K_{cat} of 0.65 ± 0.03 min⁻¹. The efficiency of Ldt_{Mav2} catalysis of nitrocefin was calculated as a K_{cat}/K_m ratio of 8.7 × 10³ min⁻¹M⁻¹ (**Figure 2B**).

• Carbapenems & cephalothin irreversibly inhibit Ldt_{Mav2}

Although β-lactams bind to and inhibit D,D-transpeptidases [35], it is not known if this antibacterial class interacts with Ldt_{Mav2}. We studied the nature of the reaction between Ldt_{Mav2} and different β-lactam subclasses using UPLC-MS. Amoxicillin and ampicillin were used as representatives of penicillin subclass; cephalothin and cefoxitin are cephalosporins; and doripenem, biapenem and tebipenem are carbapenems while faropenem is a penem. While cephalothin, imipenem, doripenem, biapenem, faropenem and tebipenem formed covalent acyl-adducts, we were unable to detect any reacted masses for amoxicillin, ampicillin, cefoxitin and meropenem (**Table 1**). Additionally, we used nitrocefin as a substrate to monitor Ldt_{Mav2} activity following treatment with the β-lactams. Hydrolysis of nitrocefin by Ldt_{Mav2} was significantly inhibited when the enzyme was treated with faropenem or tebipenem (**Figure 2C**). Cefoxitin, amoxicillin and ampicillin failed to inhibit the enzyme as nitrocefin hydrolysis occurred despite treatment with these agents.

• Kinetics of acylation of Ldt_{Mav2}

Kinetics of Ldt_{Mav2} acylation by various β-lactams was assessed in the presence of

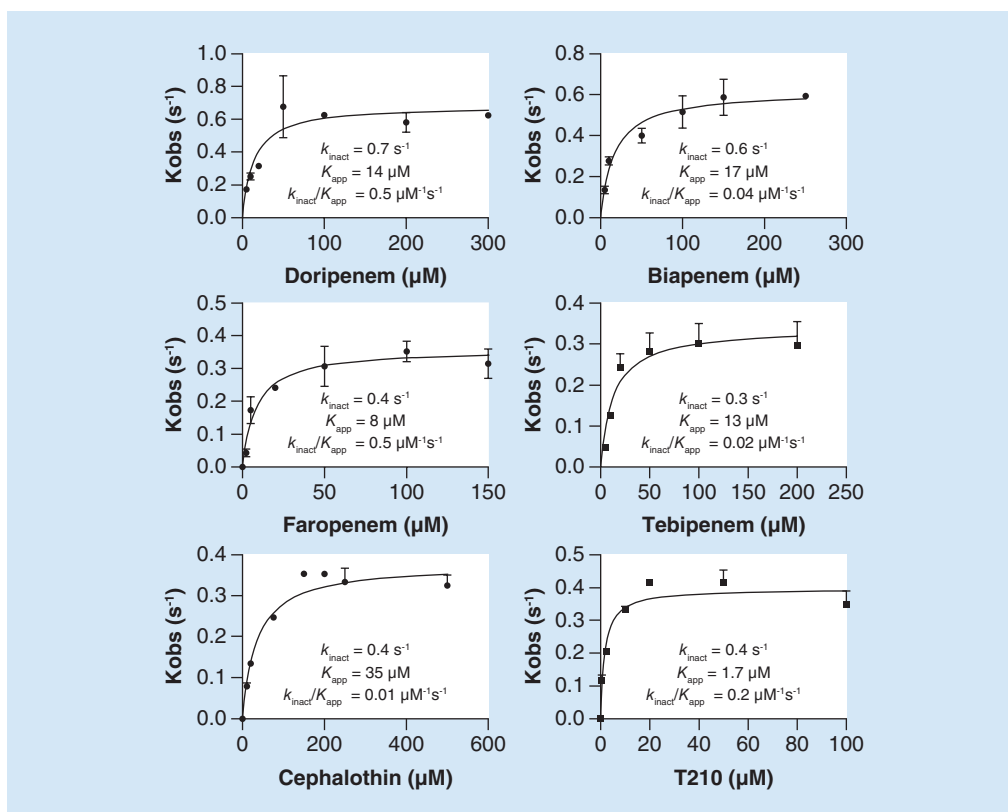


Figure 3. Kinetics of β -lactam ring opening by Ldt_{Mav2} . Plots of k_{obs} as a function of concentration of various carbapenems. Regression analysis was performed using the equation, $k_{obs} = k_{inact} [I] / K_{app} + [I]$, where k_{inact} is a first-order constant for acylenzyme formation, and K_{app} is a constant. The experiments were performed at least thrice and error represents standard deviation.

Table 2. Potency of various drugs against *Mycobacterium avium*.

Agent	MIC ₉₀ (pH 6.8)	MIC ₉₀ (pH 7.4)
Control		
Rifampin	4–8	4–8
Moxifloxacin	0.5–1	0.5–1
Clarithromycin	2–4	1–2
β-lactams		
Amoxicillin	>64	>64
Ampicillin	>64	>64
Cephalothin	>64	>64
Cefoxitin	>64	>64
Ertapenem	>64	>64
Meropenem	>64	>64
Imipenem	>64	>64
Doripenem	>64	>64
Biapenem	>64	>64
Faropenem	>64	>64
Tebipenem	2–4	4–8
Panipenem	>64	>64
T210	>64	>64

MIC₉₀ of various drugs in $\mu g/ml$ determined using two growth broths/pH as per Clinical and Laboratory Standard Guidelines [26]. T210 is a synthetic carbapenem [10].

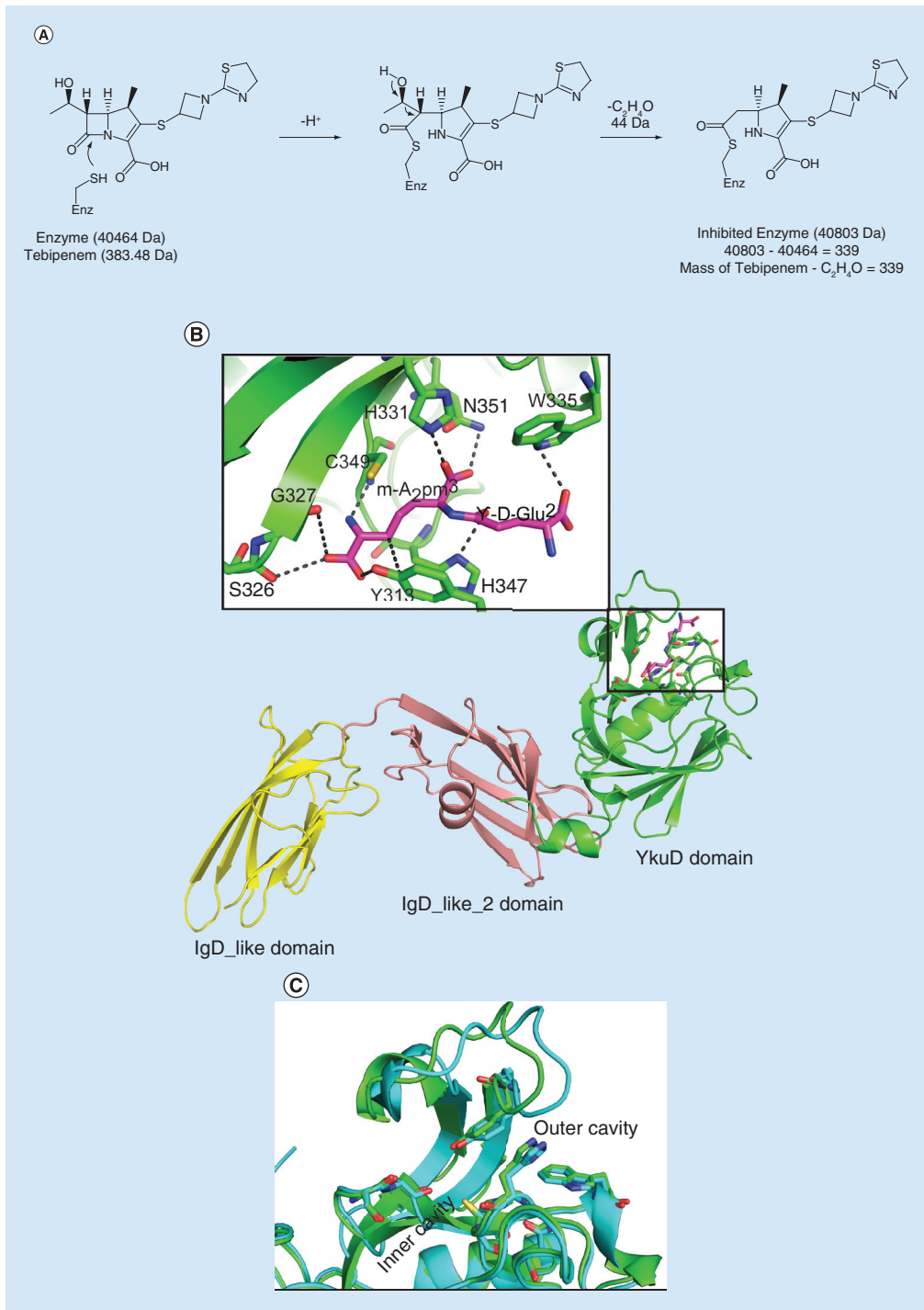


Figure 4. Ldt_{Mav2} crystal structure with peptidoglycan fragment and reaction with tebipenem. (A) Proposed mechanism of acylation of Ldt_{Mav2} by tebipenem. (B) Model of Ldt_{Mav2} with different domains, Ig-like domain (yellow), Ig-like_2 domain (salmon) and YkuD domain (green). The inset picture shows the active site of Ldt_{Mav2} with peptidoglycan substrate fragment γ -D-Glu-m-A₂pm dipeptide (pink) showing interactions with different residues. (C) Superposition of active site of Ldt_{Mav2} (green) with Ldt_{Mt2} (cyan).

increasing concentrations of β -lactams and a fixed concentration of Ldt_{Mav2} . Parameters that characterize the kinetics of reaction, k_{inact} and K_{app} were determined to be as follows: doripenem (0.7 s^{-1} and $14 \text{ }\mu\text{M}$), biapenem (0.6 s^{-1} and $17 \text{ }\mu\text{M}$), faropenem (0.4 s^{-1} and $8 \text{ }\mu\text{M}$), tebipenem (0.3 s^{-1} and $13 \text{ }\mu\text{M}$), cephalothin (0.4 s^{-1} and $35 \text{ }\mu\text{M}$) and T210 (0.4 s^{-1} and $1.7 \text{ }\mu\text{M}$; **Figure 3**). The differences in kinetics reflect changes in the chemistry of binding among the β -lactams. Amoxicillin (**Supplementary Figure 2**) and cefoxitin (data not shown) failed to inhibit Ldt_{Mav2} . The $k_{\text{inact}}/K_{\text{app}}$ ratios for doripenem ($0.5 \text{ s}^{-1} \mu\text{M}^{-1}$), biapenem ($0.04 \text{ s}^{-1} \mu\text{M}^{-1}$), faropenem ($0.5 \text{ s}^{-1} \mu\text{M}^{-1}$), tebipenem ($0.02 \text{ s}^{-1} \mu\text{M}^{-1}$), cephalothin ($0.1 \text{ s}^{-1} \mu\text{M}^{-1}$) and T210 ($0.2 \text{ s}^{-1} \mu\text{M}^{-1}$) illustrate that the efficiencies of Ldt_{Mav2} inhibition vary significantly among β -lactams.

• *M. avium* is susceptible to tebipenem but resistant to most carbapenems

We assessed a panel of β -lactams, including newer carbapenems, for activity against *M. avium* according to CLSI guidelines [26]. Tebipenem, a recently developed carbapenem with oral bioavailability and potency against *M. tuberculosis* [33], exhibited MIC_{90} of 2–4 $\mu\text{g}/\text{ml}$ at pH 6.8 and 4–8 $\mu\text{g}/\text{ml}$ in the pH 7.4 and was the only β -lactam (among those tested in this study) with a potency in the therapeutically relevant range. Other carbapenems failed to inhibit *M. avium* growth even at 64 $\mu\text{g}/\text{ml}$, the highest concentration in the range tested (**Table 2**). Next, we supplemented each of these β -lactams with clavulanate, 5 $\mu\text{g}/\text{ml}$, to assess if this β -lactamase inhibitor enhanced the susceptibility of *M. avium* to the β -lactams: the MIC of the β -lactams were not altered by the addition of clavulanate.

Incubation of Ldt_{Mav2} with tebipenem (Molecular weight; MW = 383.5 Da) produced a covalently bound acyl adduct of +339 Da (**Table 1**). The catalytic domain of Ldt_{Mt2} and the homologous domain in Ldt_{Mav2} share 96% sequence identity (**Supplementary Figure 1**). In Ldt_{Mt2} , catalytic site residues Tyr³¹⁸, His³³², His³⁵² and Cys³⁵⁴ are involved in coordination and reaction with carbapenems [36]. These residues are conserved in Ldt_{Mav2} . Based on these data, we propose the following mechanism for tebipenem adduct formation with Ldt_{Mav2} . Sulfur atom of the putative catalytic Cys³⁴⁹ makes a nucleophilic attack to the carbonyl carbon of

β -lactam ring and opens the ring (**Figure 4A**). Next, as in meropenem and tebipenem reactions with Ldt_{Mt2} [36], hydroxyethyl group at C6 is lost resulting in an adduct of +339 Da.

Carbapenems and rifampin exhibit synergy *in vitro* against *M. tuberculosis* and *M. abscessus* [27]. We hypothesized that tebipenem may exhibit synergy with rifampin against *M. avium*. Checker board assay revealed a lack of synergy between tebipenem and rifampin against this mycobacterium (**Supplementary Table 1**). Clarithromycin and moxifloxacin are commonly used for treatment of *M. avium* infections [37]. We also studied potencies of combination of clarithromycin or moxifloxacin and tebipenem; these combinations failed to exhibit synergy against *M. avium* (**Supplementary Table 1**).

• Molecular modeling of Ldt_{Mav2} with putative substrate

Structural characterization of Ldt_{Mav2} can provide further insight into its biochemical properties. Using the crystal structure of Ldt_{Mt2} with a native peptide substrate bound to its catalytic site (PDB ID: 3TUR) [30] as a template, we used homology modeling to generate a 3D model of Ldt_{Mav2} (**Figure 4B**). The modeling study suggests that overall structure of Ldt_{Mav2} is very similar to Ldt_{Mt2} . The three domains, namely IgD1, IgD2 and the YkuD domain, share very similar structures and topologies. The peptidoglycan substrate fragment γ -D-Glu-m-A₂pm dipeptide modeled through the outer cavity into the active site of Ldt_{Mav2} shows interactions with putative active-site residues including His³³¹, Trp³³⁵, Tyr³¹³, His³⁴⁷, Gly³²⁷, Asn³⁵¹ and Cys³⁴⁹. Superposition of active sites of Ldt_{Mav2} with Ldt_{Mt2} (**Figure 4C**) reveals that residues involved in substrate binding are conserved in the two proteins, suggesting a similar mechanism of catalysis and substrate specificities.

Discussion

Mycobacterial genomes encode multiple sequence orthologs with the YkuD domain (**Figure 1**). The presence of this domain is predictive of a function related to L,D-transpeptidation. While a recent study ascribed L,D-transpeptidase function to the paralogs in *M. tuberculosis* using an *in vitro* peptide cross-linking assay [38], studies that evaluated strains lacking each paralog concluded them

to be functionally nonredundant [6,14,20]. The presence of six sequence orthologs in *M. avium* genome illustrates that a significant catalytic potential of this protein class is preserved by this pathogen and perhaps also suggests their importance to this organism. In this study, we have chosen to characterize Ldt_{Mav2}, whose ortholog in *M. tuberculosis* (Ldt_{Mt2}) is the dominant L,D-transpeptidase [6].

As β -lactams are known to mimic peptide substrates of peptidoglycan biosynthesis [35] and Ldt_{Mav2} readily hydrolyzed nitrocefin, a β -lactam, it is reasonable to expect that this enzyme uses peptides or related biomolecule as its native substrate. Localization of Ldt_{Mav2} in the cell wall further suggests that similar to its ortholog Ldt_{Mt2}, it is likely anchored to the cell membrane with a single transmembrane domain and catalyzes formation of 3 \rightarrow 3 linkages in the peptidoglycan layer. We found that Ldt_{Mav2} associates with a putative Ser/Thr kinase (accession #: 48928156) possessing a homodimer interface for peptide-binding site. These two proteins exist at the site of peptidoglycan synthesis. Therefore, it is possible that the homodimer interface interacts with the stem peptide fragments generated and released during peptidoglycan metabolism and the Ser/Thr kinase domain of the protein transmits this signal to Ldt_{Mav2} or another protein. There is a precedent to this mechanism: in *Bacillus subtilis* a conserved membrane bound Ser/Thr kinase binds to peptidoglycan fragments and regulates spore germination [39]. The presence of similar levels of Ldt_{Mav2} during exponential and stationary phases of growth suggests that activity conferred by this protein is likely required constitutively by *M. avium*. Lower abundance of the protein, especially when *M. avium* is exposed to osmotic shock or tebipenem, suggests that this organism may also deploy alternative mechanisms to fortify its cell wall.

Treatment of infections with drug-resistant strains of *M. avium* pose serious challenges as the limited choices of antibacterials are often not well tolerated by patients. Therefore, availability of new and well-tolerated treatment agent that functions by inhibiting previously unexploited target can have a direct impact on the health of patients with *M. avium* infection. Consistent with the recent observations that *M. tuberculosis* L,D-transpeptidases are irreversibly inhibited by carbapenems [36,38],

we also observed that stable covalent adducts are formed between Ldt_{Mav2} and carbapenems. The efficiency of acylation of Ldt_{Mav2}, as can be described by $k_{\text{inact}}/K_{\text{app}}$, shows similar acylation kinetics among the carbapenems. However, this *in vitro* inhibition did not completely predict whole-cell activity: although all tested carbapenems except meropenem covalently acylated Ldt_{Mav2}, only tebipenem exhibited whole-cell potency against *M. avium*. Several possibilities can be speculated to account for the apparent difference between *in vitro* and whole-cell activities. We hypothesize that tebipenem may be uniquely resistant to hydrolytic activity of the native β -lactamase in *M. avium*. Equally likely is that tebipenem selectively inhibits other transpeptidases in addition to Ldt_{Mav2}. The most interesting observation with immediate clinical relevance is that tebipenem not only inhibits Ldt_{Mav2} but is also highly potent against *M. avium*. Therefore, this orally bioavailable antibacterial, from a class namely carbapenems that are generally well tolerated, has the potential to be repurposed as a much needed new therapy to treat *M. avium* infections. Studies herein, however, do not directly prove if inhibition of Ldt_{Mav2} by tebipenem is sufficient to dictate susceptibility of *M. avium* to this carbapenem.

Conclusion

M. avium genome also encodes for nonclassical transpeptidases, namely L,D-transpeptidases that generate unique 3 \rightarrow 3 cross-linkages in the peptidoglycan layer. Tebipenem, a new orally bioavailable carbapenem, not only inhibits *M. avium* L,D-transpeptidase but also potently inhibits the growth of this pathogen, thereby raises the possibility of using this potent antibiotic to treat conditions arising from infection with this organism.

Future perspective

M. avium infections are rapidly emerging as significant threats in immune-compromised patients as there are very limited antimicrobials that can be used to treat this disease. There is a critical knowledge gap in vital molecular pathways in *M. avium* that can be targeted to inhibit its growth and leverage this to identify effective antimicrobials. Here, by showing that this pathogen possesses a panel of unexploited enzymes involved in peptidoglycan synthesis and by showing that a commercially available

oral drug, namely tebipenem, not only inhibits the enzyme but also inhibits growth of this bacteria, we open the possibility to repurposing this potent antibacterial for treatment of *M. avium* infections in the future. This study focused on biochemical characterization of a putative L,D-transpeptidase Ldt_{Mav2}. Availability of molecular tools for efficient manipulation of *M. avium* would enable genetic studies of L,D-transpeptidases to furnish insight into phenotypes associated with this class of enzymes.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2016-0208

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the

principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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SUMMARY POINTS

- *Mycobacterium avium* genome encodes six proteins that are sequence paralogs of L,D-transpeptidases that are known to catalyze the nonclassical linkages, namely 3→3 linkages, in the peptidoglycan layer of mycobacteria.
- One of the enzymes studied here, Ldt_{Mav2} hydrolyzes β-lactam substrates and is also inhibited by certain classes of β-lactams, namely cephalosporins and carbapenems.
- One of the carbapenems, namely tebipenem, not only inhibits the enzyme but also has potent activity against *M. avium* raising the hope that this new orally bioavailable carbapenem may be useful to treat *M. avium* infections.

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