AAC Journals.ASM.org

In Vitro Cross-Linking of *Mycobacterium tuberculosis* Peptidoglycan by L,D-Transpeptidases and Inactivation of These Enzymes by Carbapenems

Mathilde Cordillot,[a,b,c] Vincent Dubée,[a,b,c] Sébastien Triboulet,[a,b,c] Lionel Dubost,[d,e] Arul Marie,[d,e] Jean-Emmanuel Hugonnet,[a,b,c] Michel Arthur,[a,b,c] Jean-Luc Mainardi[a,b,c,f]

Centre de Recherche des Cordeliers, LRMA, Equipe 12, Université Pierre et Marie Curie-Paris 6, UMR S 872, Paris, France[a]; INSERM, U872, Paris, France[b]; Université Paris Descartes, Sorbonne Paris Cité, UMR S 872, Paris, France[c]; Muséum National d'Histoire Naturelle, USM0502, Plateforme de Spectrométrie de Masse et de Protéomique du Muséum, Paris, France[d]; CNRS, UMR8041, Paris, France[e]; Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France[f]

The *Mycobacterium tuberculosis* peptidoglycan is cross-linked mainly by L,D-transpeptidases (LDTs), which are efficiently inactivated by a single β-lactam class, the carbapenems. Development of carbapenems for tuberculosis treatment has recently raised considerable interest since these drugs, in association with the β-lactamase inhibitor clavulanic acid, are uniformly active against extensively drug-resistant *M. tuberculosis* and kill both exponentially growing and dormant forms of the bacilli. We have purified the five L,D-transpeptidase paralogues of *M. tuberculosis* (Mt1 to -5) and compared their activities with those of peptidoglycan fragments and carbapenems. The five LDTs were functional *in vitro* since they were active in assays of peptidoglycan cross-linking (Mt5), β-lactam acylation (Mt3), or both (Mt1, Mt2, and Mt4). Mt3 was the only LDT that was inactive in the cross-linking assay, suggesting that this enzyme might be involved in other cellular functions such as the anchoring of proteins to peptidoglycan, as shown in *Escherichia coli*. Inactivation of LDTs by carbapenems is a two-step reaction comprising reversible formation of a tetrahedral intermediate, the oxyanion, followed by irreversible rupture of the β-lactam ring that leads to formation of a stable acyl enzyme. Determination of the rate constants for these two steps revealed important differences (up to 460-fold) between carbapenems, which affected the velocity of oxyanion and acyl enzyme formation. Imipenem inactivated LDTs more rapidly than ertapenem, and both drugs were more efficient than meropenem and doripenem, indicating that modification of the carbapenem side chain could be used to optimize their antimycobacterial activity.

Tuberculosis (TB) remains the second-leading infectious disease causing mortality, after AIDS, and it is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. According to the 2012 WHO report, there were 8.7 million new TB cases and 1.4 million deaths due to the disease in 2011 (1). TB treatment requires at least 6 months of chemotherapy with multiple drugs due to the poor efficacy of available antibiotics against particular forms of the *M. tuberculosis* bacilli that do not replicate (2, 3). Inappropriate use of the two first-line anti-TB drugs, isoniazid and rifampin, leads to the emergence of bacilli that are resistant to these drugs (multidrug-resistant *M. tuberculosis* [MDR-TB]), and widespread dissemination of these bacilli represents an obstacle to tuberculosis control (1). In 2011, the WHO estimated that there were 630,000 MDR-TB cases. The extensive use of second-line drugs has led to emergence of extensively drug-resistant *M. tuberculosis* (XDR-TB), which shows a very poor prognosis with an increasing mortality rate (4).

Except for bedaquiline, all drugs used to treat tuberculosis were approved more than 45 years ago, illustrating the complexity of TB drug development. β-Lactams are not used for TB treatment since *M. tuberculosis* produces a broad spectrum β-lactamase, BlaC, which inactivates all β-lactams with various efficiencies (5). However, BlaC is irreversibly inactivated by clavulanic acid, and the combination of this β-lactamase inhibitor with β-lactams of the carbapenem class has been reported to be bactericidal *in vitro* (6, 7). The combination is active against both exponentially growing *M. tuberculosis* and nonreplicating forms of the bacilli (7). Furthermore, the combination is uniformly active against XDR strains (7).

Antibiotics of the β-lactam family inhibit the last step of peptidoglycan polymerization. In most bacteria, the drugs inactivate the essential D,D-transpeptidase activity of classical penicillin-binding proteins (PBPs) (8, 9). These enzymes cross-link glycan chains by forming 4⟶3 peptide bonds connecting residues at the fourth and third positions of stem peptides (Fig. 1). Only 20% of the peptidoglycan cross-links are of the 4⟶3 type in mycobacteria, indicating that classical PBPs may only have a minor role in peptidoglycan polymerization (10). The majority of the cross-links are of the 3⟶3 type and are formed by L,D-transpeptidases (LDTs) (Fig. 1). PBPs and LDTs are structurally unrelated (11), contain active-site serine and cysteine residues (12), and use stem pentapeptide and tetrapeptide as the acyl donor substrate (12), respectively. Since pentapeptide is assembled in the cytoplasm, formation of the tetrapeptide donor substrate of LDTs requires a D,D-carboxypeptidase activity for cleaving the terminal D-Ala residue of peptidoglycan precursors (13, 14).

β-Lactam antibiotics inactivate peptidoglycan transpeptidases by forming a covalent adduct with the enzyme active-site residue.

Received 30 July 2013 **Returned for modification** 24 August 2013
Accepted 7 September 2013
Published ahead of print 16 September 2013
Address correspondence to Jean-Luc Mainardi, jean-luc.mainardi@crc.jussieu.fr, or Michel Arthur, michel.arthur@crc.jussieu.fr.

doi:10.1128/AAC.01663-13

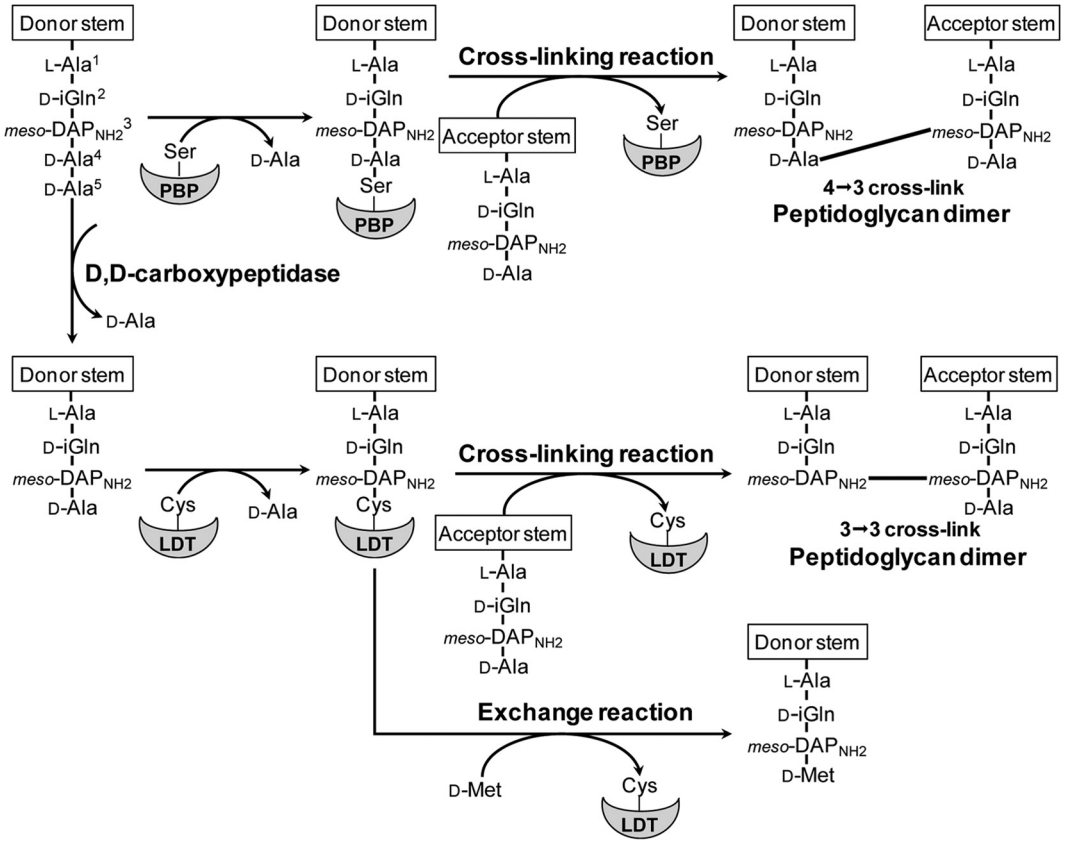

FIG 1 Reactions catalyzed by *Mycobacterium tuberculosis* D,D-transpeptidases (PBPs) and L,D-transpeptidases (LDTs).

The reaction is analogous to the first step of the transpeptidation reaction that leads to enzyme acylation by the donor stem peptide (15). LDTs are efficiently inactivated by a single class of β-lactams, the carbapenems, such as imipenem, meropenem, ertapenem, and doripenem (16) (Fig. 2). LDTs are likely to be the essential targets of carbapenems in *M. tuberculosis*, since these enzymes are responsible for formation of a substantial majority of the cross-links in both the exponential and stationary phases of growth (10, 17). However, the D,D-carboxypeptidase activity of PBPs is an additional target, since tetrapeptide production is essential to gener-

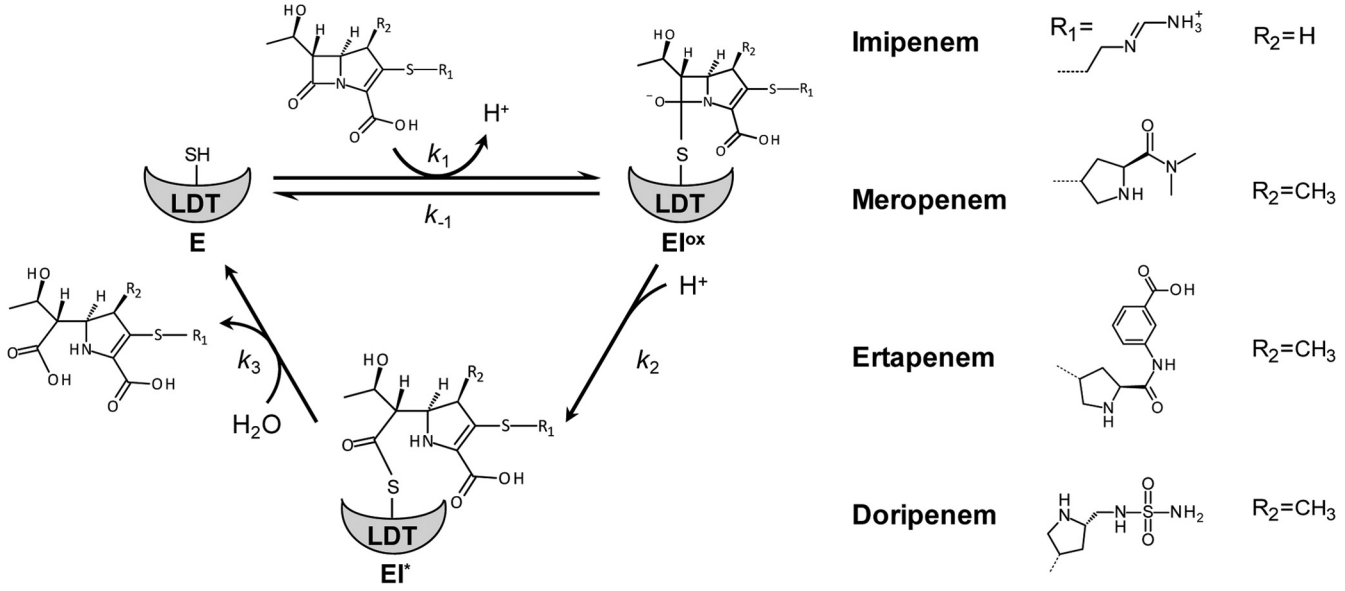

FIG 2 Reaction schemes for LDT inactivation by carbapenems. E, free enzyme form; EI^{ox}, oxyanion; EI*, acyl enzyme. SH, sulfhydryl of the catalytic cysteine.

TABLE 1 Oligonucleotides used for amplification and cloning of L,D-transpeptidase genes

L,D-Transpeptidase (strain; accession no.) and amplicon[a]	Oligonucleotide[b]
Mt2 (Rv2518c; CAA16014.1)	
C	5′AACATATGGCCGATCTGCTGGTGCC (for)
D	5′TTCTCGAGTTACGCCTTGGCGTTACCGG (rev)
Mt3 (Rv1433; CAB09251.1)	
A	5′TATGCAGTCTTACGGGTTCGCCGT (for)
	5′GTTATTCCTGCACAATGACCGGGT (rev)
B	5′TGCAGTCTTACGGGTTCGCCGT (for)
	5′TCGAGTTATTCCTGCACAATGACCGGGT (rev)
Mt4 (Rv0192; CAB09732.1)	
C	5′CATATGCCACACTGGGCTGAAGAACG (for)
D	5′CTCGAGTTAGATCTGCCAGTCCTGGGCACC (rev)
Mt5 (Rv0483; CAB00944.1)	
A	5′TATGGCCGGCAAAGTGACCAAGCT (for)
	5′GTTACCCACCCGGTCCGTTAGTAG (rev)
B	5′TGGCCGGCAAAGTGACCAAGCTGG (for)
	5′TCGAGTTACCCACCCGGTCCGTTAGTAG (rev)

[a] For cloning, amplicons A and B were mixed, denatured, and renatured to generate heteroduplexes with cohesive ends.
[b] Initiation and stop codons are in italic. For Mt2 and Mt4, oligonucleotides C and D contained NdeI and XhoI sites (underlined). for, forward; rev, reverse.

ate the tetrapeptide donor substrate of LDTs (12, 14, 17). Classical D,D-transpeptidases may also be essential, although these enzymes have a minor contribution to peptidoglycan cross-linking.

The chromosome of *M. tuberculosis* strain H37Rv encodes five LDTs paralogues. Ldt_{Mt1} and Ldt_{Mt2} are functional in an *in vitro* peptidoglycan cross-linking assay and are thought to have distinct functions *in vivo* (10, 18). Ldt_{Mt2} is essential for virulence in a mouse model of acute infection (18), whereas Ldt_{Mt1} may have a role in adaptation to the nonreplicative state of the bacilli (10). The roles of the three remaining LDTs have not been investigated. To further characterize the targets of carbapenems in *M. tuberculosis*, we have purified the five L,D-transpeptidase paralogues and compared the *in vitro* activities of the enzymes with respect to peptidoglycan dimer formation and acylation by carbapenems.

MATERIALS AND METHODS

Production and purification of L,D-transpeptidases. Recombinant plasmids for production of the soluble form of Ldt_{Mt1} (Rv0116c) containing an N-terminal hexahistidine tag have been previously described (19). For production of Ldt_{Mt2}, Ldt_{Mt3}, Ldt_{Mt4}, andLdt_{Mt5}, portions of the genes from *M. tuberculosis* H37Rv were amplified by PCR using oligonucleotides described in Table 1. Heteroduplexes containing cohesive ends were ligated with vector pET-TEV digested with NdeI plus XhoI as previously described (19). *Escherichia coli* BL21(DE3) harboring recombinant plasmids was grown at 37°C with vigorous shaking in 2 liters of brain heart infusion broth (Difco) containing kanamycin (50 μg/ml) to an optical density at 600 nm of 0.9. Isopropyl-β-D-thiogalactopyranoside was added (0.5 mM), and incubation was continued for 18 h at 16°C. LDTs were purified from clarified lysates by affinity chromatography on Ni^{2+}-nitrilotriacetate-agarose resin (Sigma) and by size exclusion chromatography (Superdex 75 HL26/60 column; GE HealthCare) in 100 mM sodium phosphate buffer (pH 6.4) containing 300 mM NaCl. L,D-Transpeptidases were concentrated by ultrafiltration (Amicon Ultra-4 centrifugal filter devices; Millipore) to a final concentration of ca. 1.5 mg/ml and stored at −65°C in the same buffer.

Mass spectrometry analyses of L,D-transpeptidase acylation by carbapenems. The formation of drug-enzyme adducts was tested by incubating L,D-transpeptidases (20 μM) with carbapenems (100 μM) at 20°C in 100 mM sodium phosphate buffer (pH 6.0). Five microliters of acetonitrile and 1 μl of 1% formic acid were added, and the reaction mixture was directly injected into the mass spectrometer (Qstar Pulsar I; Applied Biosystems) at a flow rate of 0.05 ml/min (acetonitrile, 50%; water, 49.5%; formic acid, 0.5% [by volume]). Spectra were acquired in the positive mode as previously described (16).

Kinetics of L,D-transpeptidase inactivation by carbapenems. Fluorescence kinetic data were acquired with a stopped-flow apparatus (RX-2000; Applied Biophysics) coupled to a spectrofluorometer (Cary Eclipse; Varian) in 100 mM sodium phosphate (pH 6.0) at 10°C. Kinetic constants for LDT acylation by carbapenems were calculated as previously described (19, 20). The rates of hydrolysis of acyl enzymes were determined by spectrophotometry in sodium phosphate buffer (100 mM; pH 6.0) at 20°C in a Cary 100 spectrophotometer (Cary 100-Bio; Varian) as previously described (19).

Mass spectrometry analyses of transpeptidation reaction products. The disaccharide tetrapeptide containing amidated *meso*-diaminopimelic acid (GlcNAc–MurNGlyc–L-Ala^1–D-iGln^2–*meso*-$Dap_{NH2}{}^3$–D-Ala^4) was purified from *Mycobacterium abscessus* strain CIP104536T, and the concentration was determined by amino acid analysis after acid hydrolysis (21). *In vitro* formation of muropeptide dimers by LDTs (10 μM) was tested in 10 μl of 20 mM sodium phosphate buffer (pH 6.4) containing 60 mM NaCl and 4 mM disaccharide tetrapeptide. The reaction mixture was incubated for 2 h at 37°C and analyzed by electrospray mass spectrometry in the positive mode (Qstar Pulsar I; Applied Biosystems) as previously described (22). D-Met (1 mM) was added to the reaction mixture to assay the exchange of D-Ala^4 by D-Met. The transpeptidases assayed were also tested with disaccharide pentapeptide GlcNAc–MurNGlyc–L-Ala^1–D-iGln^2–*meso*-$Dap_{NH2}{}^3$–D-Ala^4–D-Ala^5 (440 μM), which was purified from *M. abscessus* strain CIP104536T.

RESULTS

Covalent inactivation of L,D-transpeptidases by carbapenems. LDTs were independently incubated with four carbapenems (imipenem, meropenem, ertapenem, and doripenem), and formation of covalent adducts was assayed by electrospray mass spectrometry (Table 2). Mass increments corresponding to the masses of the antibiotics were detected, indicating that adducts were generated by formation of a thioester bond between the sulfhydryl group of the L,D-transpeptidase active-site cysteine and the carbonyl group of the carbapenem β-lactam ring (Fig. 2) (16). Formation of covalent adducts was not detected for Ldt_{Mt5}, whereas the four remaining L,D-transpeptidases were acylated by each of the four carbapenems (Table 2). Thus, only four of the five *M. tuberculosis* L,D-transpeptidases are inactivated by carbapenems.

Kinetics of enzyme acylation. We have previously shown that L,D-transpeptidase inactivation by carbapenems is a two-step reaction (Fig. 2) (20, 23). The first step is reversible and leads to formation of a tetrahedral intermediate, the oxyanion (EI^{ox}). The second step is irreversible and leads to formation of the acyl enzyme (EI*). Fluorescence kinetics indicated that Ldt_{Mt1}, Ldt_{Mt2}, Ldt_{Mt3}, and Ldt_{Mt4}, but not Ldt_{Mt5}, were inactivated by carbapenems (Table 3), in agreement with mass spectrometry analyses (Table 2). The rate constants k_1 and k_2 for formation of the oxyanion and acyl enzyme, respectively, were determined by spectrofluorometry according to previously described procedures (20, 23). The k_2/K_{app} ratio provides an evaluation of the overall efficacy of the reaction based on estimates of the disappearance of free enzyme. Important variations in the velocity of the acylation reaction were detected between LDTs (Table 3). The dynamic range of k_2/K_{app} ratios reached 375 for inactivation of Ldt_{Mt1} and Ldt_{Mt2} by ertapenem. For imipenem, Ldt_{Mt3} was the most rapidly inactivated L,D-transpeptidase ($k_2/K_{app} = 1.16 \pm 0.03\ \mu M^{-1}\ min^{-1}$), due to high rate constants for both oxyanion formation (k_1 =

TABLE 2 Masses of acyl enzymes

L,D-Transpeptidase (residues[a])	Carbapenem (mass, Da)	Avg mass (Da)[b]	
		Calculated	Observed
Mt1 (32–251)	None	26,092.3	26,092.6
	Imipenem (299.3)	26,391.6	26,391.8
	Meropenem (383.5)	26,475.8	26,475.4
	Ertapenem (475.5)	26,567.8	26,567.5
	Doripenem (420.1)	26,512.4	26,512.6
Mt2 (55–408)	None	40,236.8	40,236.7
	Imipenem	40,536.1	40,538.0
	Meropenem	40,620.3	40,622.4
	Ertapenem	40,712.3	40,714.8
	Doripenem	40,656.9	40,657.3
Mt3 (32–271)	None	28,160.6	28,161.1
	Imipenem	28,459.9	28,460.9
	Meropenem	28,544.1	28,544.5
	Ertapenem	28,636.1	28,637.4
	Doripenem	28,580.7	28,581.5
Mt4 (1–366)	None	41,178.2	41,178.9
	Imipenem	41,477.5	41,478.0
	Meropenem	41,561.7	41,561.8
	Ertapenem	41,653.7	41,653.3
	Doripenem	41,598.3	41,597.7
Mt5 (50–451)	None	45,140.3	45,140.2
	Imipenem	45,439.6	ND
	Meropenem	45,523.8	ND
	Ertapenem	45,615.8	ND
	Doripenem	45,560.4	ND

[a] Portion of the *M. tuberculosis* H37Rv L,D-transpeptidase present in recombinant proteins produced in *E. coli*. Each L,D-transpeptidase contained an additional N-terminal 6× His tag (MHHHHHHENLYFQGHM). The N-terminal methionine was not present in the purified proteins.
[b] Mass of enzyme or acyl enzyme. ND, not detected.

$0.92 \pm 0.03\ \mu M^{-1}\ min^{-1}$) and acylation ($k_2 = 10.1 \pm 0.2\ min^{-1}$). Ldt_{Mt1} and Ldt_{Mt4} were also efficiently inactivated by imipenem ($k_2/K_{app} = 0.61 \pm 0.02$ and $0.31 \pm 0.01\ \mu M^{-1}\ min^{-1}$, respectively). Inactivation of Ldt_{Mt2} by this antibiotic was less efficient ($k_2/K_{app} = 0.081 \pm 0.002\ \mu M^{-1}\ min^{-1}$) due to slow formation of the oxyanion ($k_1 = 0.00067 \pm 0.00002\ \mu M^{-1}\ min^{-1}$). For the three remaining carbapenems, Ldt_{Mt1} was more efficiently inactivated than Ldt_{Mt2}, Ldt_{Mt3}, and Ldt_{Mt4}. Among these three enzymes, Ldt_{Mt3} was acylated more efficiently by meropenem than Ldt_{Mt4}, but similar values were observed for ertapenem and doripenem. For all carbapenems, the efficiency of acylation was the lowest for Ldt_{Mt2}.

Important variations in the velocity of the acylation reaction were also detected between carbapenems. The dynamic range of k_2/K_{app} ratios reached 460 for inactivation of Ldt_{Mt4} by imipenem and meropenem. Based on the k_2/K_{app} ratios (Table 3), the overall efficacies of the four carbapenems could be ranked as imipenem > ertapenem > meropenem = doripenem.

***In vitro* cross-linking of purified peptidoglycan fragments.** Substrates of the L,D-transpeptidases were prepared by isolation of *M. abscessus* sacculi, treatment with muramidases, and purification of the resulting disaccharide peptide fragments by reverse-phase high-pressure liquid chromatography (rpHPLC). Incubation of Ldt_{Mt1}, Ldt_{Mt2}, Ldt_{Mt4}, and Ldt_{Mt5} with a disaccharide tetrapeptide resulted in formation of peptidoglycan dimers containing a 3⟶3 cross-link (Fig. 1 and Table 4). Formation of disaccharide tripeptide by cleavage of the *meso*-$DAP_{NH2}{}^3$–D-Ala^4 peptide bond was not detected, indicating that the four L,D-transpeptidases did not display L,D-carboxypeptidase activity (data not shown). The four enzymes are specific for a donor containing a stem tetrapeptide, since formation of dimers was not observed with disaccharide pentapeptide GlcNAc–MurNGlyc–L-Ala–D-

TABLE 3 Kinetic constants for *M. tuberculosis* L,D-transpeptidase inactivation by carbapenems

L,D-Transpeptidase and kinetic constant	Value (mean ±SD) for:			
	Imipenem	Meropenem	Ertapenem	Doripenem
Mt1				
$k_1 \times 10^3\ (\mu M^{-1}\ min^{-1})$	460 ± 20	64 ± 2	930 ± 60	130 ± 10
$k_2 \times 10^3\ (min^{-1})$	1,810 ± 20	3,600 ± 80	2,660 ± 50	470 ± 20
$k_2/K_{app} \times 10^3\ (\mu M^{-1}\ min^{-1})$	610 ± 20	75 ± 2	1,350 ± 50	89 ± 4
$k_3 \times 10^3\ (min^{-1})$	≤0.12	1.04 ± 0.09	1.01 ± 0.12	0.58 ± 0.09
Mt2				
$k_1 \times 10^3\ (\mu M^{-1}\ min^{-1})$	67 ± 5	0.67 ± 0.02	4.0 ± 0.1	0.85 ± 0.04
$k_2 \times 10^3\ (min^{-1})$	11,200 ± 700	660 ± 40	1,010 ± 30	580 ± 30
$k_2/K_{app} \times 10^3\ (\mu M^{-1}\ min^{-1})$	81 ± 2	0.48 ± 0.01	3.6 ± 0.1	0.56 ± 0.02
$k_3 \times 10^3\ (min^{-1})$	≤0.06	0.72 ± 0.03	0.48 ± 0.08	0.34 ± 0.05
Mt3				
$k_1 \times 10^3\ (\mu M^{-1}\ min^{-1})$	920 ± 30	17 ± 1	87 ± 3	10.2 ± 0.5
$k_2 \times 10^3\ (min^{-1})$	10,100 ± 200	6,800 ± 700	6,500 ± 600	4,500 ± 300
$k_2/K_{app} \times 10^3\ (\mu M^{-1}\ min^{-1})$	1,160 ± 30	21 ± 1	80 ± 2	12 ± 1
$k_3 \times 10^3\ (min^{-1})$	≤0.12	1.42 ± 0.26	1.02 ± 0.06	0.38 ± 0.01
Mt4				
$k_1 \times 10^3\ (\mu M^{-1}\ min^{-1})$	250 ± 10	5.0 ± 0.3	31 ± 3	NA[a]
$k_2 \times 10^3\ (min^{-1})$	9,900 ± 300	80 ± 20	18,000 ± 1,000	NA
$k_2/K_{app} \times 10^3\ (\mu M^{-1}\ min^{-1})$	310 ± 10	0.67 ± 0.024	37 ± 1	9.7 ± 1.7
$k_3 \times 10^3\ (min^{-1})$	≤0.06	5.6 ± 0.6	3.9 ± 0.7	0.61 ± 0.27

[a] NA, not applicable (constants k_1 and k_2 could not be determined since fluorescence kinetics were monophasic).

TABLE 4 Observed monoisotopic masses of L,D-transpeptidase reaction products[a]

L,D-Transpeptidase	Calculated mass (Da) of reaction product	
	Peptidoglycan dimer (1,817.66 Da)	Exchange of D-Ala by D-Met (1,013.35 Da)
Mt1	1,817.81	1,013.44
Mt2	1,817.81	1,013.43
Mt3	ND[b]	ND
Mt4	1,817.81	1,013.43
Mt5	1,817.79	1,013.43

[a] L,D-Transpeptidases were incubated with GlcNAc–MurNGlyc–L-Ala–D-iGln–*meso*DAP_{NH2}–D-Ala alone (peptidoglycan dimer) or with D-Met (exchange of D-Ala by D-Met).
[b] ND, not detected.

iGln–*meso*DAP_{NH2}–D-Ala–D-Ala (data not shown). Incubation of the four enzymes with disaccharide tetrapeptide and D-methionine resulted in the exchange of D-Ala^4 by D-Met (Fig. 1 and Table 4). Formation of peptidoglycan dimers and exchange of D-Ala^4 by D-Met were not observed with Ldt_{Mt3}.

DISCUSSION

The increasing incidence of MDR and XDR tuberculosis indicates that there is an urgent need for new anti-TB drugs (24). In this context, LDTs are attractive targets, since the *M. tuberculosis* peptidoglycan contains a majority of 3→3 cross-links formed by these enzymes (10, 17). To investigate peptidoglycan inhibition by β-lactams, we have characterized five *M. tuberculosis* L,D-transpeptidase paralogues with respect to peptidoglycan dimer formation and acylation by carbapenems. We have obtained functional forms of the five L,D-transpeptidases, since each enzyme was active in the cross-linking assay (Mt5), the β-lactam acylation assay (Mt3), or both (Mt1, Mt2, and Mt4).

Ldt_{Mt3} was not active in the peptidoglycan cross-linking assay. In *E. coli*, two L,D-transpeptidases catalyze peptidoglycan cross-linking, whereas three additional paralogues anchor the Braun lipoprotein to peptidoglycan (25, 26). This observation suggests that Ldt_{Mt3} may perform as-yet-unknown cross-linking reactions in *M. tuberculosis*.

Ldt_{Mt5} was not inactivated by carbapenems but was active in the peptidoglycan cross-linking assay. The antibacterial activity of carbapenems suggests that Ldt_{Mt5} cannot compensate for the activity of the other L,D-transpeptidases. In *Streptococcus pneumoniae* and *E. coli*, it has been established that multiple D,D-transpeptidases are essential for peptidoglycan polymerization (9).

Carbapenems also inactivate D,D-carboxypeptidase DacB and prevent formation of tetrapeptide stems in the *M. tuberculosis* peptidoglycan (17). Although D,D-carboxypeptidases are considered unessential in *E. coli* (27), these enzymes may be required for peptidoglycan polymerization in *M. tuberculosis*, since they generate the tetrapeptide donor stem for formation of 3→3 cross-links by LDTs (17). In agreement, we have shown here that the four LDTs that are active in the cross-linking assay are specific for donor substrates containing a stem tetrapeptide. Ldt_{fs} from *Enterococcus faecalis* is the only known L,D-transpeptidase that uses both tetrapeptide and pentapeptide stems as acyl donors (22). In *Enterococcus faecium*, stem tetrapeptides are produced in the cytoplasm by metallo-D,D-carboxypeptidase DdcY, which cleaves the C-terminal D-Ala residue of UDP-MurNAc pentapeptide (14). The genome of *M. tuberculosis* does not code for a DdcY homologue, indicating that tetrapeptide stems are produced only by D,D-carboxypeptidases belonging to the PBP family in this species.

We have previously shown that ertapenem and imipenem are the most efficient β-lactams for *in vitro* inactivation of Ldt_{Mt1}, with high rate constants for formation of the oxyanion (k_1) and acylation (k_2) (19). The meropenem side chain moderately impaired drug binding (lower k_1), whereas that of doripenem had an additional unfavorable impact on the acylation step (lower k_2) (19). The analyses of Ldt_{Mt2}, Ldt_{Mt3}, and Ldt_{Mt4} reported in the present study revealed greater differences between carbapenems. For these enzymes, inactivation was more rapid with imipenem than with ertapenem, whereas acylation by meropenem and doripenem was very slow. These results indicate that modification of the side chains of carbapenems could be used to optimize the antibacterial activity of these drugs. Detection of multiple LDTs with highly diverse inactivation kinetics also suggests that combination therapy including two β-lactams may lead to synergism.

ACKNOWLEDGMENTS

This work was supported by the seventh Framework Program of the European Community, Project Open Collaborative Model for Tuberculosis Lead Optimization (ORCHID, 261378). M.C. was the recipient of a scholarship from Paris Descartes University, V.D. was supported by a Poste d'Accueil, Institut National de la Santé et de la Recherche Médicale, and S.T. was supported by ORCHID.

We thank D. Blanot for the determination of muropeptide concentrations by amino acid analysis.

REFERENCES

1. **Anonymous.** 2012. Tuberculosis 2011. WHO report. WHO, Geneva, Switzerland.
2. **Dick T.** 2001. Dormant tubercle bacilli: the key to more effective TB chemotherapy? J. Antimicrob. Chemother. **47:**117–118.
3. **Wayne LG, Sohaskey CD.** 2001. Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu. Rev. Microbiol. **55:**139–163.
4. **Kliiman K, Altraja A.** 2009. Predictors of extensively drug-resistant pulmonary tuberculosis. Ann. Intern. Med. **150:**766–775.
5. **Wang F, Cassidy C, Sacchettini JC.** 2006. Crystal structure and activity studies of the *Mycobacterium tuberculosis* beta-lactamase reveal its critical role in resistance to beta-lactam antibiotics. Antimicrob. Agents Chemother. **50:**2762–2771.
6. **Hugonnet JE, Blanchard JS.** 2007. Irreversible inhibition of the *Mycobacterium tuberculosis* beta-lactamase by clavulanate. Biochemistry **46:**11998–12004.
7. **Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE 3rd Blanchard JS.** 2009. Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. Science **323:**1215–1218.
8. **Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P.** 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol. Rev. **32:**234–258.
9. **Zapun A, Contreras-Martel C, Vernet T.** 2008. Penicillin-binding proteins and beta-lactam resistance. FEMS Microbiol. Rev. **32:**361–385.
10. **Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L, Mainardi JL.** 2008. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. J. Bacteriol. **190:**4360–4366.
11. **Biarrotte-Sorin S, Hugonnet JE, Delfosse V, Mainardi JL, Gutmann L, Arthur M, Mayer C.** 2006. Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. J. Mol. Biol. **359:**533–538.
12. **Mainardi JL, Fourgeaud M, Hugonnet JE, Dubost L, Brouard JP, Ouazzani J, Rice LB, Gutmann L, Arthur M.** 2005. A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. J. Biol. Chem. **280:**38146–38152.
13. **Mainardi JL, Morel V, Fourgeaud M, Cremniter J, Blanot D, Legrand R, Fréhel C, Arthur M, van Heijenoort J, Gutmann L.** 2002. Balance between two transpeptidation mechanisms determines the expression of

beta-lactam resistance in *Enterococcus faecium*. J. Biol. Chem. **277:**35801–35807.
14. **Sacco E, Hugonnet JE, Josseaume N, Cremniter J, Dubost L, Marie A, Patin D, Blanot D, Rice LB, Mainardi JL, Arthur M.** 2010. Activation of the L,D-transpeptidation peptidoglycan cross-linking pathway by a metallo-D,D-carboxypeptidase in *Enterococcus faecium*. Mol. Microbiol. **75:**874–885.
15. **Tipper DJ, Strominger JL.** 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc. Natl. Acad. Sci. U. S. A. **54:**1133–1141.
16. **Mainardi JL, Hugonnet JE, Rusconi F, Fourgeaud M, Dubost L, Nguekam Moumi A, Delfosse V, Mayer C, Gutmann L, Rice LB, Arthur M.** 2007. Unexpected inhibition of peptidoglycan L,D-transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. J. Biol. Chem. **282:**30414–30422.
17. **Kumar P, Arora K, Lloyd JR, Lee IY, Nair V, Fischer E, Boshoff HI, Barry CE 3rd.** 2012. Meropenem inhibits D,D-carboxypeptidase activity in *Mycobacterium tuberculosis*. Mol. Microbiol. **86:**367–381.
18. **Gupta R, Lavollay M, Mainardi JL, Arthur M, Bishai WR, Lamichhane G.** 2010. The *Mycobacterium tuberculosis* protein Ldt_{Mt2} is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. Nat. Med. **16:**466–469.
19. **Dubée V, Triboulet S, Mainardi J, Ethève-Quelquejeu M, Marie A, Dubost L, Hugonnet JE, Arthur M.** 2012. Inactivation of *Mycobacterium tuberculosis* L,D-transpeptidase Ldt_{Mt1} by carbapenems and cephalosporins. Antimicrob. Agents Chemother. **56:**4189–4195.
20. **Triboulet S, Arthur M, Mainardi JL, Veckerlé C, Dubée V, Nguekam Moumi A, Gutmann L, Rice LB, Hugonnet JE.** 2011. Inactivation kinetics of a new target of beta-lactam antibiotics. J. Biol. Chem. **286:**22777–22784.
21. **Auger G, van Heijenoort J, Mengin-Lecreulx D, Blanot D.** 2003. A MurG assay which utilises a synthetic analogue of lipid I. FEMS Microbiol. Lett. **219:**115–119.
22. **Magnet S, Arbeloa A, Mainardi JL, Hugonnet JE, Fourgeaud M, Dubost L, Marie A, Delfosse V, Mayer C, Rice LB, Arthur M.** 2007. Specificity of L,D-transpeptidases from gram-positive bacteria producing different peptidoglycan chemotypes. J. Biol. Chem. **282:**13151–13159.
23. **Triboulet S, Dubée V, Lecoq L, Bougault C, Mainardi JL, Rice LB, Ethève-Quelquejeu M, Gutmann L, Marie A, Dubost L, Hugonnet JE, Simorre JP, Arthur M.** 2013. Kinetic features of L,D-transpeptidase inactivation critical for β-lactam antibacterial activity. PLoS One **8:**e67831. doi:10.1371/journal.pone.0067831.
24. **Mainardi JL, Hugonnet JE, Gutmann L, Arthur M.** 2011. Fighting resistant tuberculosis with old compounds: the carbapenem paradigm. Clin. Microbiol. Infect. **17:**1755–1756.
25. **Magnet S, Bellais S, Dubost L, Fourgeaud M, Mainardi JL, Petit-Frère S, Marie A, Mengin-Lecreulx D, Arthur M, Gutmann L.** 2007. Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. J. Bacteriol. **189:**3927–3931.
26. **Magnet S, Dubost L, Marie A, Arthur M, Gutmann L.** 2008. Identification of the L,D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. J. Bacteriol. **190:**4782–4785.
27. **Denome SA, Elf PK, Henderson TA, Nelson DE, Young KD.** 1999. *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. J. Bacteriol. **181:**3981–3993.