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In most bacteria, the essential targets of β-lactam antibiotics are the D,D-transpeptidases that catalyze the last step of peptidoglycan polymerization by forming 4→3 cross-links. The peptidoglycan of *Clostridium difficile* is unusual since it mainly contains 3→3 cross-links generated by L,D-transpeptidases. To gain insight into the characteristics of *C. difficile* peptidoglycan cross-linking enzymes, we have purified the three putative *C. difficile* L,D-transpeptidases paralogues, Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3}, which have been previously identified by sequence analysis. The catalytic activity of the three proteins was assayed with a disaccharide-tetrapeptide purified form the *C. difficile* cell wall. Ldt_{Cd2} and Ldt_{Cd3} catalyzed the formation of 3→3 cross-links (L,D-transpeptidase activity), the hydrolysis of the C-terminal D-Ala residue of the disaccharide-tetrapeptide substrate (L,D-carboxypeptidase activity), and the exchange of the C-terminal D-Ala by D-Met. Ldt_{Cd1} only displayed L,D-carboxypeptidase activity. Mass spectrometry analyses indicated that Ldt_{Cd1} and Ldt_{Cd2} were acylated by β-lactams belonging to the carbapenem (imipenem, meropenem, and ertapenem), cephalosporin (ceftriaxone), and penicillin (ampicillin) classes. Acylation of Ldt_{Cd3} by these β-lactams was not detected. The acylation efficacy of Ldt_{Cd1} and Ldt_{Cd2} was higher for the carbapenems (480 to 6,600 M^{-1} s^{-1}) than for ampicillin and ceftriaxone (3.9 to 82 M^{-1} s^{-1}). In contrast, the efficacy of hydrolysis of β-lactams by Ldt_{Cd1} and Ldt_{Cd2} was higher for ampicillin and ceftriaxone than for imipenem. These observations indicate that Ldt_{Cd1} and Ldt_{Cd2} are only inactivated by β-lactams of the carbapenem class due to a combination of rapid acylation coupled to the stability of the resulting covalent adducts.

Clostridium difficile is an anaerobic Gram-positive spore-forming bacterium responsible for 15 to 25% of post-antibiotic diarrhea and more than 95% of pseudomembranous colitis (1). It is the first cause of nosocomial infectious diarrhea in adults (2). Dysbiosis due to antibiotics is

thought to be the main cause of *C. difficile*-associated diseases (3). Antibiotics that promote these infections are active on *C. difficile* highlighting the likely importance of spore formation. The recent evolution of *C. difficile*-associated diseases is worrisome, due to the emergence and rapid spread of the virulent *C. difficile* clone 027 (4).

Metronidazole *per os* is the first line treatment for non-severe infections (5). The emergence of isolates with intermediate susceptibility may compromise the efficacy of metronidazole (6). Vancomycin *per os* is used in severe infections (5), but this treatment is associated with a risk of emergence of vancomycin-resistant enterococci. A new antibiotic, fidaxomicin, is recommended in recurrent infections (5), but this drug has not been evaluated in severe infections (7, 8). Fidaxomicin has not been used for a sufficient time period to evaluate the risk of resistance emergence and therapeutic options remain limited for *C. difficile*-associated diseases.

The structure of cell wall peptidoglycan is unusual in *C. difficile* since the majority (70%) of the cross-links are of the 3→3 type formed by L,D-transpeptidases (LDTs) (Fig. 1A) (9). This feature is only shared with mycobacteria (10, 11), as the peptidoglycan from all other bacterial species that have been studied mainly or exclusively contain 4→3 cross-links formed by the D,D-transpeptidase activity of classical penicillin-binding proteins (PBPs) (12) (Fig. 1B). LDTs and PBPs are structurally unrelated, harbor different catalytic residues (Cys or Ser, respectively), and use distinct acyl donors for the cross-linking reaction (tetrapeptide or pentapeptide, respectively) (13-15). PBPs are potentially inhibited by all classes of β-lactams whereas LDTs are only inhibited by carbapenems (16, 17).

The genome of *C. difficile* encodes three L,D-transpeptidase paralogues, designated Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3} (Fig. 1C), potentially involved in the formation of 3→3 cross-links (70%), and four high-molecular weight PBPs, potentially involved in the formation of the remaining cross-links (4→3 type; 30%) (9). The inactivation of the genes ldt_{Cd1} or ldt_{Cd2} has

been reported to result in significant decrease in the abundance of 3→3 cross-links and in the overall peptidoglycan reticulation but the growth rate was not affected. These observations indicate that Ldt_{Cd1} and Ldt_{Cd2} have redundant cross-linking activities (9). In the presence of ampicillin, the relative proportions of 3→3 cross-links increased as expected from the lack of inhibition of LDTs by β-lactams belonging to the penam (penicillin) class (9).

To date, the characteristics of *C. difficile* LDTs has only been indirectly inferred from the study of mutants deficient in the production of Ldt_{Cd1} or Ldt_{Cd2} and from the impact of ampicillin on the relative abundance of 3→3 and 4→3 cross-links (9). To gain insight into the catalytic properties of LDT paralogues from *C. difficile,* we have purified Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3} and determined their peptidoglycan cross-linking activity. We have also investigated the inhibition of the three LDTs by β-lactams.

RESULTS AND DISCUSSION

Purification of soluble Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3}. Two forms of Ldt_{Cd1} were produced in *E. coli* and purified, which comprised the entire protein without the N-terminal transmembrane segment or the catalytic domain only (Fig. 1). The catalytic domain was used for mass spectrometry analyses of protein-β-lactam adducts, while the larger form of the protein was used for the transpeptidation, stopped-flow spectrofluorometry, and spectrophotometry assays due to its higher solubility. For Ldt_{Cd2}, all experiments were performed with the catalytic domain. The catalytic domain of Ldt_{Cd3} was not soluble but this issue was solved by purifying the entire protein, which does not harbor any putative transmembrane segment in contrast to Ldt_{Cd1} and Ldt_{Cd2}.

Transpeptidase and carboxypeptidase activities of Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3}. The activities of the three recombinant LDTs were assayed with a disaccharide-tetrapeptide substrate purified from the cell-wall peptidoglycan of *C. difficile* (Fig. 1). Purified LDTs and

the disaccharide-tetrapeptide were incubated for 3 h and formation of the products was assayed by mass spectrometry. Incubation of Ldt_{Cd2} and Ldt_{Cd3} with the disaccharide-tetrapeptide substrate resulted in the formation of a dimer and a disaccharide-tripeptide indicating that these enzymes display both L,D-transpeptidase (LDT) and L,D-carboxypeptidase (LD-CPase) activities (Fig. 1A and Table 1). The dimer contained a 3→3 cross-link connecting two disaccharide-tripeptides (Table 1). This may imply two mutually non-exclusive reaction schemes involving both the LDT and LD-CPase activities of Ldt_{Cd2} and Ldt_{Cd3}. For the first reaction scheme, the disaccharide-tripeptide generated by the LD-CPase activity is used as the acyl-acceptor substrate to generate the dimer (as depicted in Fig. 1A). For the second reaction scheme, the cross-linking reaction involves an acyl acceptor containing a tetrapeptide stem and D-Ala^4 is cleaved off from the dimer by the LD-CPase activity.

Formation of an acyl-enzyme is a common intermediary for the LDT and LD-CPase activities (Fig. 1A). Prior analyses of the LDT from *E. faecium* (Ldt_{fm}) have shown that the acyl-enzyme may also react with free amino acids of the D configuration, such as D-Met, or glycine (15). The net result is the replacement of D-Ala^4 by D-Met or Gly at the extremity of a tetrapeptide stem (exchange reaction, Fig. 1A). Both Ldt_{Cd2} and Ldt_{Cd3} catalyzed this reaction (Table 1).

The remaining L,D-transpeptidase, Ldt_{Cd1}, did not catalyze formation of 3→3 cross-links or the exchange of D-Ala^4 by D-Met (Table 1). However, Ldt_{Cd1} was purified in an active form since it cleaved the DAP^3-D-Ala^4 bond to form a disaccharide-tripeptide. Thus, the disaccharide-tetrapeptide was used as an acyl donor but not as an acyl acceptor by Ldt_{Cd1}.

Acyl-enzymes formed by Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3} with β-lactams. Ldt_{Cd3} did not form any covalent adduct with the five β-lactams that were tested (data not shown). Ldt_{Cd3} was purified in a catalytically active form since it catalyzed cross-link formation (above,

Table 1). In *M. tuberculosis*, one of the five L,D-transpeptidase paralogues (Ldt_{Mt5}) similarly catalyzes formation of peptidoglycan dimers *in vitro* in the absence of detectable acylation by β-lactams (18).

For Ldt_{Cd1} and Ldt_{Cd2}, mass spectrometry analysis revealed the formation of acyl-enzymes for representatives of three classes of β-lactams (Table 2). For carbapenems, the mass of the acyl-enzymes (EI*) corresponded to the mass of the proteins plus the mass of the β-lactam indicating the presence of the entire drugs in the covalent adducts (Fig. 2). Acylation of the L,D-transpeptidases by the cephalosporin ceftriaxone led to the loss of the R_2 side-chain (acyl-enzyme EI**). The acyl-enzyme containing the complete drug (EI*) was not detected suggesting a concerted mechanism involving simultaneous acylation and loss of the R_2 side-chain, as previously described (17, 19). Incubation of LDTs with ampicillin led to the formation of two acyl-enzymes containing the complete drug (EI*) or a drug fragment following cleavage of the C^5-C^6 bond of the β-lactam ring (EI**). This unexpected cleavage of a carbon-carbon bond has been previously reported for L,D-transpeptidases (17) and a D,D-carboxypeptidase (20).

Kinetics of acylation of Ldt_{Cd1} and Ldt_{Cd2} by β-lactams. Stopped-flow spectrofluorometry was used to compare the efficacy of inactivation of the L,D-transpeptidases by β-lactams (Table 3 and Fig. 3). The method was developed for Ldt_{fm} from *E. faecium* (21) and applied to the characterization of the L,D-transpeptidases from *M. tuberculosis* (18). The fluorescence kinetics obtained with these enzymes is bi-phasic (see Fig. 3C for the example of Ldt_{fm}). In the first phase, the fluorescence of the Trp residues of the enzymes is quenched due to reversible formation of an oxyanion. In the second phase, the fluorescence intensity increases due to the formation of the acyl-enzyme to the detriment of the oxyanion. The bi-phasic nature of the fluorescence kinetics is accounted for by the large variations in the fluorescence of the three forms of the enzyme, which is maximum for the apo

form (E), minimum for the oxyanion (EI^{ox}), and intermediate for the acyl-enzyme (EI*). Surprisingly, monophasic kinetics were observed for inactivation of *C. difficile* Ldt_{Cd1} and Ldt_{Cd2} by carbapenems, as exemplified in Fig. 3A by the data obtained for inactivation of Ldt_{Cd2} by imipenem. This monophasic behavior may be accounted for by the lack of accumulation of the oxyanion or by the absence of a significant difference between the fluorescence quantum yields of the oxyanion and of the acyl-enzyme (21). Since this monophasic behavior prevented the independent evaluation of the kinetic parameters for the formation of the oxyanion and of the acylenzyme, we determined the overall efficacy of inactivation of the L,D-transpeptidases by carbapenems (Fig. 3B and Table 3), as previously described (17). The efficacy of acylation of Ldt_{Cd1} and Ldt_{Cd2} by ampicillin and ceftriaxone was *ca.* 10- to 50-fold lower than the efficacy of acylation of these L,D-transpeptidases by carbapenems. Overall the efficacy of acylation of the L,D-transpeptidases from *C. difficile* (this work), *M. tuberculosis* (18), *and E. faecium* (17) were in the same order of magnitudes and all enzymes displayed specificity for carbapenems. Incubation of Ldt_{Cd3} with carbapenems did not result in variations in fluorescence intensity (data not shown) in agreement with the absence of detection of any covalent adduct by mass spectrometry (above).

Kinetics of β-lactam hydrolysis by Ldt_{Cd1} and Ldt_{Cd2}. The turnover for hydrolysis of β-lactams by the L,D-transpeptidases of *C. difficile* (Table 4) showed that the covalent adducts formed by acylation of these enzymes by carbapenems were stable. Half-lives greater than 150 min were deduced from the observed turnovers for the hydrolysis of the carbapenems, which were smaller than 8×10^{-5} s^{-1} (see reference 17 for the calculation). The acyl-enzyme formed with ampicillin and Ldt_{Cd2} was the least stable, as previously described (17), with a half-life of 2.0 min. This value indicates that Ldt_{Cd2} is poorly inhibited by ampicillin although the low turnover for hydrolysis implies that the enzyme is unlikely to contribute to resistance

by acting as β-lactamase. In comparison to the L,D-transpeptidases from *E. faecium* and *M. tuberculosis* (17, 18), the turnovers for hydrolysis of β-lactams by *C. difficile* L,D-transpeptidases did not reveal any striking difference since the acyl-enzyme stability was the highest for carbapenems, the lowest for penams (penicillin), and intermediary for cephems (cephalosporins).

Conclusions. Peltier *et al*. (9) have previously shown that the peptidoglycan of *C. difficile* contains an unusually high (73%) content of 3→3 cross-links in comparison to all other firmicutes that have been submitted to peptidoglycan structure analysis, typically <10% or 0 % depending upon the presence (*e.g. E. faecium*) or absence (*e.g. Staphylococcus aureus*) of genes encoding members of the LDT family in their genome (22). Deletion of the genes encoding Ldt_{Cd1} and Ldt_{Cd2}, alone or in combination, was associated with a decrease in the proportion of muropeptide dimers containing a 3→3 cross links from 41% for the wild-type strain to 19%, 26%, and 13%, respectively (9). This observation provided evidence that Ldt_{Cd1} and Ldt_{Cd2} form 3→3 cross links *in vivo* (9). For an unknown reason, the proportion of the 4→3 cross links was also reduced, albeit to a lower extent (from 15% for the wild-type strain to 11%, 11%, and 12% for deletion of genes ldt_{Cd1}, ldt_{Cd2}, and ldt_{Cd1} plus ldt_{Cd2}, respectively) (9). Consequently, the deletions of ldt_{Cd1}, ldt_{Cd2}, and ldt_{Cd1} plus ldt_{Cd2} led to a decrease in the proportion of dimers (from 57% for the wild-type strain to 30%, 37%, and 34%, respectively), with a moderate impact on the relative proportions of 3→3 cross links among dimers, which were only reduced from 73% for the wild-type strain to 64%, 70% and 66%, respectively (9). These observations indicate that increased formation of 4→3 cross links by the PBPs did not compensate for the absence of Ldt_{Cd1} and Ldt_{Cd2}, resulting in a less cross-linked peptidoglycan.

The detection of 3→3 cross-links in the double mutant lacking Ldt_{Cd1} and Ldt_{Cd2} revealed the existence of a third L,D-transpeptidase in *C. difficile* (9). The corresponding gene

was tentatively identified as CD3007 but attempts for its inactivation were unsuccessful (9). In the current study, purification of the product of CD3007 showed that this enzyme is indeed functional as a peptidoglycan cross-linking enzyme and the corresponding protein was therefore designated as Ldt_{Cd3}. Together, the results obtained by the inactivation of the *ldt* genes by Peltier *et al*. (9) and by the characterization of purified enzymes in the current study clearly indicate that the three LDTs of *C. difficile* have redundant functions, at least partially, as observed for PBPs in most bacteria. However, there are discrepancies between the two approaches. In particular, formation of 3→3 cross-links by purified Ldt_{Cd1} was not detected *in vitro* although deletion of the corresponding gene led to a reduction of the proportion of dimers containing 3→3 cross-links in the peptidoglycan layer. This may indicate that Ldt_{Cd1} does not catalyze the cross-linking reactions with substrates consisting of an isolated disaccharide peptidoglycan fragment.

MATERIALS AND METHODS

Plasmid construction. The oligonucleotide primers used to amplify portions of the ldt_{Cd1} and ldt_{Cd2} genes and the entire ldt_{Cd3} gene are described in the Supplementary Table 1. Each ldt_{Cd} gene was independently amplified with two sets of primers (A1 plus A2 and B1 plus B2). The amplicons A1-A2 and B1-B2 were purified by agarose gel electrophoresis, mixed, denatured, and annealed. Since the extremity of the amplicons A1-A2 and B1-B2 differed by the presence of the additional bases TA and TCGA (underlined in the Supplementary Table 1), one of the two putative heteroduplexes contained cohesive ends compatible with the cohesive ends generated by the restriction endonucleases NdeI (CA↓TATG) and XhoI (C↓TCGAG), respectively. The heteroduplexes were ligated with the vector pET-TEV digested by NdeI plus XhoI and the sequence of the insert in the resulting recombinant plasmids was confirmed.

Production and purification of L,D-transpeptidases. The vector pET-TEV (our laboratory collection) is a derivative of pET28a (Novagen) conferring resistance to kanamycin and enabling to obtain translational fusions consisting of a polyhistidine tag followed by a TEV protease cleavage site (MHHHHHHENLYFQGHM), as previously used for production of recombinant L,D-transpeptidases (17). *E. coli* BL21(DE3) harboring the recombinant pET-TEV (17) derivatives encoding Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3} were grown in brain-heart infusion broth (Difco) containing kanamycin (50 µg/ml) at 37°C until the optical density at 600 nm reached 0.8. Isopropyl-β-D-1-thiogalactopyranoside (0.5 mM) was added and the incubation was continued for 18 h at 16°C. Bacteria were collected by centrifugation (5,200 x *g*; 4°C), resuspended in 100 mM sodium phosphate buffer (pH 6.4) containing 300 mM NaCl (buffer A), and lysed by sonication. Cell debris was removed by centrifugation (17,400 x *g*; 4°C). The clarified lysate was filtered and LDTs were purified by nickel-affinity chromatography (Ni-NTA resin, Sigma-Aldrich) and size-exclusion chromatography (Superdex column 75 HL 26/60; GE HealthCare) in buffer A. LDTs were concentrated by ultrafiltration (Amicon Ultra-4 centrifugal filter devices; Millipore) to a final concentration of 1 mg/ml and stored at -65°C in buffer A. LDTs were purified in the absence of a reducing agent, as previously described (*e.g*. ref 18), as the active-site residue of this family of enzymes is not readily oxidized.

Peptidoglycan cross-linking assay. The disaccharide-tetrapeptide used as the substrate (Fig. 1) was purified from the peptidoglycan of *C. difficile* strain CD630. Briefly, bacteria were grown in brain heart infusion broth at 35°C under anaerobic conditions during 48 h without stirring. Peptidoglycan was extracted by the boiling SDS procedure, treated with pronase and trypsin, and digested with mutanolysin and lysozyme (23). The resulting muropeptides (disaccharide-peptides) were purified by *rp*HPLC (23), the disaccharide-tetrapeptide was identified by mass spectrometry (23), and the concentration was determined by amino acid analysis after acid hydrolysis (24).

Synthesis of peptidoglycan cross-links by LDTs (10 µM) was determined in 10 µL of 15 mM sodium-phosphate buffer (pH 7.0) containing 100 µM of disaccharide-tetrapeptide. Incubation was performed at 37°C for 3 h. The exchange assay was performed in the same conditions in the presence of D-Met (1 mM). The products of the reactions catalyzed by the LDTs were identified by electrospray mass spectrometry in the positive mode (Qstar Pulsar I, Applied Biosystem), as previously described (23).

Mass spectrometry analyses of L,D-transpeptidase acylation by β-lactams. L,D-transpeptidases (10 µM) were incubated with β-lactams (100 µM) in a volume of 5 µl for 60 min at 20°C in 100 mM sodium-phosphate buffer (pH 6.0). The reaction mixture was injected into the mass spectrometer (Qstar Pulsar I, Applied Biosystem) at a flow rate of 0.05 ml/min (acetonitrile 50%, water 49.5%, formic acid 0.5 %; per volume). Spectra were acquired in the positive mode, as previously described (16).

Kinetics of L,D-transpeptidase inactivation by β-lactams. Fluorescence 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 (17, 21). Hydrolysis of β-lactams by the LDTs was determined by spectrophotometry in sodium phosphate buffer (100 mM; pH 6.0) at 20°C in a Cary 100 spectrophotometer (Varian) (17, 21). The variation in the molar extinction coefficient resulting from opening of the β-lactam ring has been previously determined for carbapenems (-7,100 M^{-1} cm^{-1} at 299 nm), ceftriaxone (-9,600 M^{-1} cm^{-1} at 265 nm), and ampicillin (-700 M^{-1} cm^{-1} at 240 nm) (17).

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Transparency declaration

We have no competing interests to declare.

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TABLE 1 Monoisotopic mass (Dalton) of L,D-transpeptidase (LDT) reaction products

	Reaction product (calculated mass)		
	Tri[a]	Dimer[a]	Tetra (D-Met)[b]
LDT	(828.36)	(1,638.71)	(959.40)
Ldt_{Cd1}	828.37	ND	ND
Ldt_{Cd2}	828.36	1,638.71	959.38
Ldt_{Cd3}	828.37	1,638.66	959.40

[a] LDTs were incubated with the disaccharide-tetrapeptide GlcN-MurNAc-L-Ala-γ-D-Glu-DAP-D-Ala leading to the formation of a disaccharide-tripeptide (L,D-carboxypeptidase activity) and of a dimer containing a 3→3 cross-link (L,D-transpeptidase activity).

[b] LDTs were incubated with the disaccharide-tetrapeptide and D-Met leading to the exchange of D-Ala^4 by D-Met.

ND, not detected; Tri, GlcN-MurNAc-L-Ala-γ-D-Glu-DAP; Tetra(D-Met), GlcN-MurNAc-L-Ala-γ-D-Glu-DAP-D-Met.

TABLE 2 Mass of L,D-transpeptidases (LDTs) and of acyl-enzymes formed with various β-lactams

LDT		Enzyme or acyl-enzyme		Mass	
β-lactam	(Mass)	(expected mass increase)		Calculated	Observed
Ldt_{Cd1}		E	(NA)	15,753.5	15,753.6
Imipenem	(299.3)	EI*	(299.3)	16,052.8	16,053.0
Meropenem	(383.5)	EI*	(383.5)	16,137.0	16,137.3
Ertapenem	(475.5)	EI*	(475.5)	16,229.0	16,229.2
Ceftriaxone	(554.6)	EI**	(395.4)	16,148.9	16,148.9
Ampicillin	(349.4)	EI*	(349.4)	16,102.9	ND
Ampicillin	(349.4)	EI**	(190.2)	15,943.7	15,943.9
Ldt_{Cd2}		E	(NA)	16,430.5	16,430.7
Imipenem	(299.3)	EI*	(299.3)	16,729.8	16,730.3
Meropenem	(383.5)	EI*	(383.5)	16,814.0	16,814.3
Ertapenem	(475.5)	EI*	(475.5)	16,906.0	16,906.3
Ceftriaxone	(554.6)	EI**	(395.4)	16,825.9	16,826.5
Ampicillin	(349.4)	EI*	(349.4)	16,779.9	16,780.0
Ampicillin	(349.4)	EI**	(190.2)	16,620.7	16,620.6

All values are average masses in Dalton. E, designate the native form of the enzymes observed in the absence of any antibiotic. EI* and EI** designate two acyl-enzymes formed with the same β-lactam (ampicillin or ceftriaxone) due to cleavage of the drugs after the acylation reaction. ND, not detected. Acylation was partial for ceftriaxone and ampicillin but complete for carbapenems.

TABLE 3 Acylation efficacy of *C. difficile* L,D-transpeptidases by β-lactams

	Acylation efficacy (M^{-1} s^{-1}) [a]	
β-lactam	Ldt_{Cd1}	Ldt_{Cd2}
Ampicillin	3.9 ± 0.6	66 ± 3
Ceftriaxone	82 ± 20	40 ± 1
Imipenem	3,100 ± 400	6,600 ± 100
Meropenem	1,100 ± 400	480 ± 20
Ertapenem	3,200 ± 500	1,030 ± 20

[a] Values are the mean ± standard error from the linear regression for the kinetic parameter k_2/K_{app}.

~~[b] The acylation of the LDTs was not detected by spectrofluorometry. ND, not detected.~~

TABLE 4 Hydrolysis of β-lactams by *C. difficile* L,D-transpeptidases

	Hydrolysis turnover x 10^6 (s^{-1})[a]	
β-lactam	Ldt_{Cd1}	Ldt_{Cd2}
Ampicillin	< 1,000	5,800 ± 400
Ceftriaxone	160 ± 10	180 ± 15
Imipenem	< 180	< 180
Meropenem	30 ± 5	48 ± 7
Ertapenem	40 ± 3	79 ± 13

[a] Values are the mean ± standard error from the linear regression for the turnover

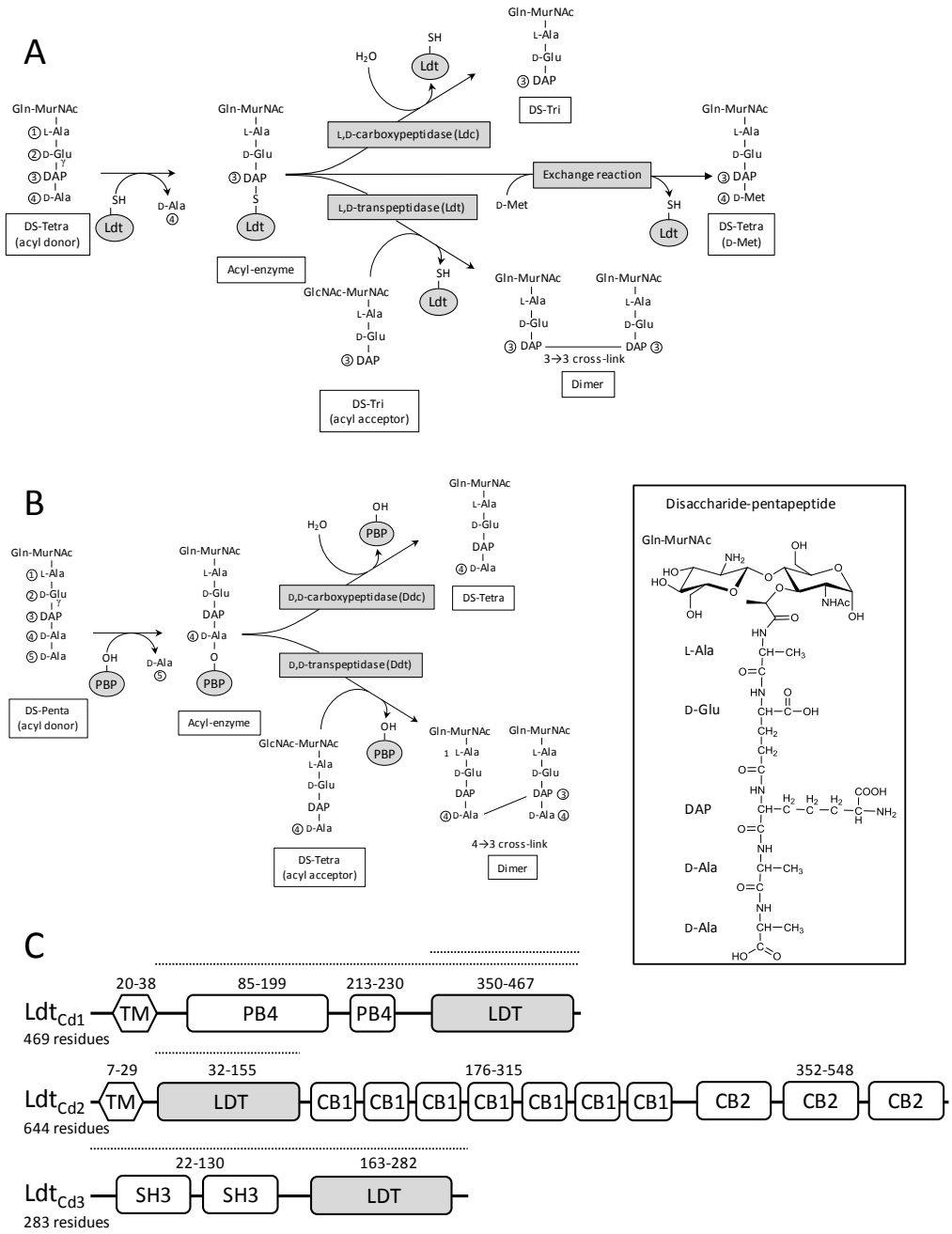


FIG 1 Peptidoglycan cross-linking in C. *difficile*. The inset shows the developed structure of the disaccharide-pentapeptide subunit of *C. difficile*. (A) Reactions catalyzed by L,D-transpeptidases (Ldt). (B) Reactions catalyzed by penicillin-binding proteins (PBPs). (C) Domain composition of Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3}. Numbers refer to the boundaries of the domains. Dashed lines indicate the portions of Ldt_{Cd1} (residues 39-469 and 350-469), Ldt_{Cd2} (30-156), and Ldt_{Cd3} (1-256) present in recombinant proteins produced in *E. coli*. These portions of the proteins comprise (i) the entire protein without the putative transmembrane segment or the catalytic L,D-transpeptidase domain for $Ldt_{Cd1,}$ (ii) the catalytic domain for Ldt_{Cd2}, and (iii) the entire protein for Ldt_{Cd3}. The recombinant proteins contained an additional vector-encoded N-terminal 6 x His Tag followed by a TEV cleavage site (MGSSHHHHHHSSGENLYFQGHM). Domain identification is based on sequences similarities in the absence of functional data. CB1 and CB2, cell wall binding type 1 and 2 domains, respectively; Ldt, catalytic L,D-transpeptidase domain; PB4, protein-binding pattern type 4; SH3, bacterial domain SH3; TM, transmembrane segment.

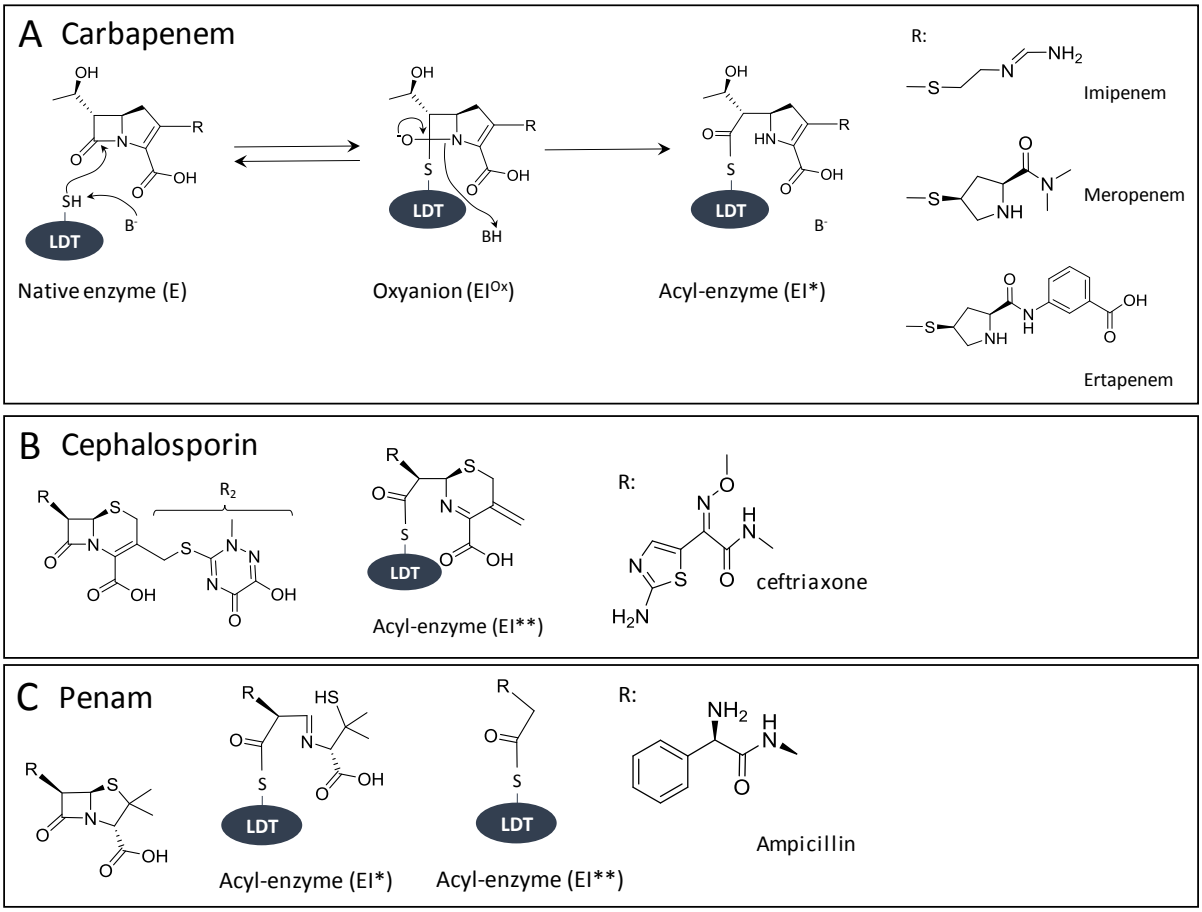
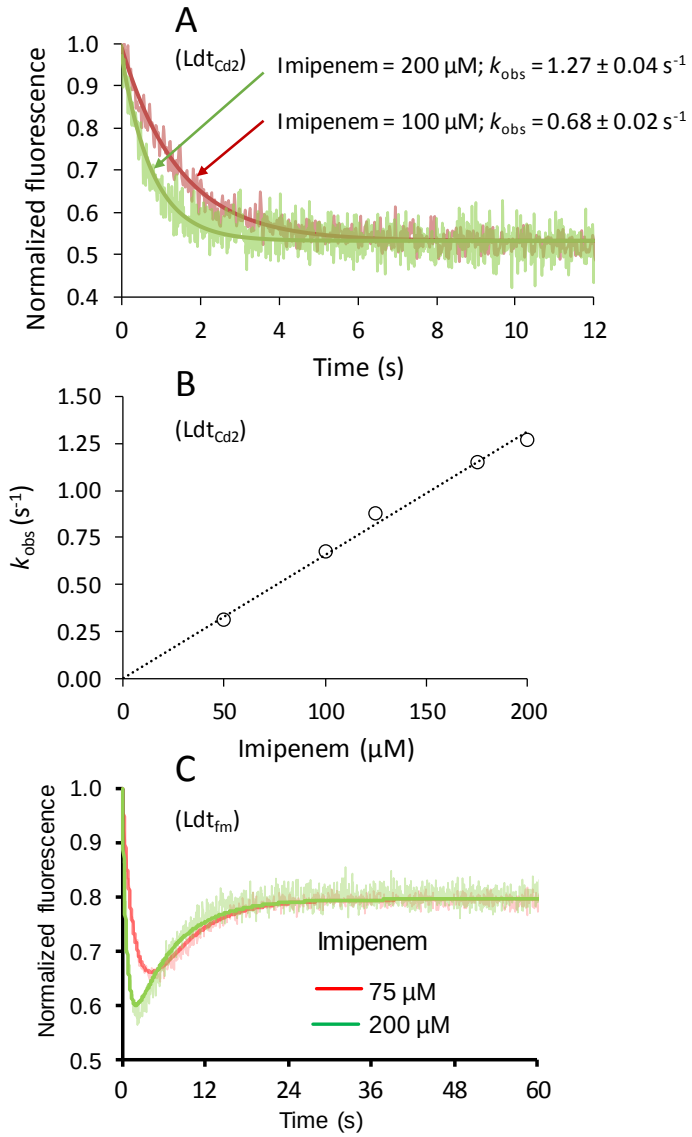


FIG 2 Acyl-enzymes formed by *C. difficile* L,D-transpeptidases with β-lactams. (A) Two-step reaction leading to irreversible inactivation of an L,D-transpeptidase (LDT) by a carbapenem. (B) and (C) Acylenzymes formed with a cephalosporin and a penam, respectively.



397 **FIG 3** Kinetics of acylation of *C. difficile* L,D-transpeptidases Ldt$_{Cd2}$ by imipenem. (A)
398 Examples of stopped-flow fluorescence kinetics obtained with Ldt$_{Cd2}$ (10 µM) and two
399 concentrations of imipenem (100 and 200 µM). The rate constant k_{obs} was determined by
400 fitting data to exponential decay. (B) The rate constant k_{obs} increased linearly with the
401 concentration of imipenem. The slope provided an estimate of the efficacy of enzyme
402 acylation (6,600 ± 100 M^{-1} s^{-1}). (C) Examples of stopped-flow fluorescence kinetics obtained
403 with Ldt$_{fm}$ (10 µM) and two concentrations of imipenem (from reference 17).