

**Peptidoglycan cross-linking activity of  
L,D-transpeptidases from Clostridium difficile and  
inactivation of these enzymes by  $\beta$ -lactams**

Laetitia Sütterlin, Zainab Edo, Jean-Emmanuel Hugonnet, Jean-Luc  
Mainardi, Michel Arthur

► **To cite this version:**

Laetitia Sütterlin, Zainab Edo, Jean-Emmanuel Hugonnet, Jean-Luc Mainardi, Michel Arthur. Peptidoglycan cross-linking activity of L,D-transpeptidases from Clostridium difficile and inactivation of these enzymes by  $\beta$ -lactams. Antimicrobial Agents and Chemotherapy, American Society for Microbiology, 2017, <10.1128/AAC.01607-17>. <hal-01634876>

**HAL Id: hal-01634876**

**<https://hal.sorbonne-universite.fr/hal-01634876>**

Submitted on 14 Nov 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Peptidoglycan cross-linking activity of L,D-transpeptidases from *Clostridium difficile* and**  
2 **inactivation of these enzymes by  $\beta$ -lactams**

3 Laetitia Sütterlin, Zainab Edo, Jean-Emmanuel Hugonnet, Jean-Luc Mainardi, and Michel  
4 Arthur

5

6 INSERM UMRS 1138, Sorbonne Universités, UPMC Univ Paris 06; Sorbonne Paris Cité,  
7 Université Paris Descartes, Université Paris Diderot; Centre de Recherche des Cordeliers,  
8 75006 Paris, France; Service de Microbiologie, Assistance Publique-Hôpitaux de Paris,  
9 Hôpital Européen Georges Pompidou, Paris, France

10

11 Running title: *C. difficile* L,D-transpeptidases

12 Keywords:  $\beta$ -lactam, carbapenems, *Clostridium difficile*, L,D-transpeptidases, peptidoglycan.

13 Corresponding author: Michel Arthur. [michel.arthur@crc.jussieu.fr](mailto:michel.arthur@crc.jussieu.fr).

14

15

16 In most bacteria, the essential targets of  $\beta$ -lactam antibiotics are the D,D-transpeptidases  
17 that catalyze the last step of peptidoglycan polymerization by forming 4 $\rightarrow$ 3 cross-links.  
18 The peptidoglycan of *Clostridium difficile* is unusual since it mainly contains 3 $\rightarrow$ 3 cross-  
19 links generated by L,D-transpeptidases. To gain insight into the characteristics of *C.*  
20 *difficile* peptidoglycan cross-linking enzymes, we have purified the three putative *C.*  
21 *difficile* L,D-transpeptidases paralogues, Ldt<sub>Cd1</sub>, Ldt<sub>Cd2</sub>, and Ldt<sub>Cd3</sub>, which have been  
22 previously identified by sequence analysis. The catalytic activity of the three proteins  
23 was assayed with a disaccharide-tetrapeptide purified from the *C. difficile* cell wall.  
24 Ldt<sub>Cd2</sub> and Ldt<sub>Cd3</sub> catalyzed the formation of 3 $\rightarrow$ 3 cross-links (L,D-transpeptidase  
25 activity), the hydrolysis of the C-terminal D-Ala residue of the disaccharide-tetrapeptide  
26 substrate (L,D-carboxypeptidase activity), and the exchange of the C-terminal D-Ala by  
27 D-Met. Ldt<sub>Cd1</sub> only displayed L,D-carboxypeptidase activity. Mass spectrometry analyses  
28 indicated that Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub> were acylated by  $\beta$ -lactams belonging to the  
29 carbapenem (imipenem, meropenem, and ertapenem), cephalosporin (ceftriaxone), and  
30 penicillin (ampicillin) classes. Acylation of Ldt<sub>Cd3</sub> by these  $\beta$ -lactams was not detected.  
31 The acylation efficacy of Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub> was higher for the carbapenems (480 to 6,600  
32 M<sup>-1</sup> s<sup>-1</sup>) than for ampicillin and ceftriaxone (3.9 to 82 M<sup>-1</sup> s<sup>-1</sup>). In contrast, the efficacy of  
33 hydrolysis of  $\beta$ -lactams by Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub> was higher for ampicillin and ceftriaxone  
34 than for imipenem. These observations indicate that Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub> are only  
35 inactivated by  $\beta$ -lactams of the carbapenem class due to a combination of rapid  
36 acylation coupled to the stability of the resulting covalent adducts.

37

38 *Clostridium difficile* is an anaerobic Gram-positive spore-forming bacterium responsible for  
39 15 to 25% of post-antibiotic diarrhea and more than 95% of pseudomembranous colitis (1). It  
40 is the first cause of nosocomial infectious diarrhea in adults (2). Dysbiosis due to antibiotics is

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

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

53 The structure of cell wall peptidoglycan is unusual in *C. difficile* since the majority  
54 (70%) of the cross-links are of the 3→3 type formed by L,D-transpeptidases (LDTs) (Fig. 1A)  
55 (9). This feature is only shared with mycobacteria (10, 11), as the peptidoglycan from all  
56 other bacterial species that have been studied mainly or exclusively contain 4→3 cross-links  
57 formed by the D,D-transpeptidase activity of classical penicillin-binding proteins (PBPs) (12)  
58 (Fig. 1B). LDTs and PBPs are structurally unrelated, harbor different catalytic residues (Cys  
59 or Ser, respectively), and use distinct acyl donors for the cross-linking reaction (tetrapeptide  
60 or pentapeptide, respectively) (13-15). PBPs are potentially inhibited by all classes of β-  
61 lactams whereas LDTs are only inhibited by carbapenems (16, 17).

62 The genome of *C. difficile* encodes three L,D-transpeptidase paralogues, designated  
63 Ldt<sub>Cd1</sub>, Ldt<sub>Cd2</sub>, and Ldt<sub>Cd3</sub> (Fig. 1C), potentially involved in the formation of 3→3 cross-links  
64 (70%), and four high-molecular weight PBPs, potentially involved in the formation of the  
65 remaining cross-links (4→3 type; 30%) (9). The inactivation of the genes *ldt*<sub>Cd1</sub> or *ldt*<sub>Cd2</sub> has

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

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

77

## 78 RESULTS AND DISCUSSION

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

88 **Transpeptidase and carboxypeptidase activities of Ldt<sub>Cd1</sub>, Ldt<sub>Cd2</sub>, and Ldt<sub>Cd3</sub>.** The  
89 activities of the three recombinant LDTs were assayed with a disaccharide-tetrapeptide  
90 substrate purified from the cell-wall peptidoglycan of *C. difficile* (Fig. 1). Purified LDTs and

91 the disaccharide-tetrapeptide were incubated for 3 h and formation of the products was  
92 assayed by mass spectrometry. Incubation of  $Ldt_{Cd2}$  and  $Ldt_{Cd3}$  with the disaccharide-  
93 tetrapeptide substrate resulted in the formation of a dimer and a disaccharide-tripeptide  
94 indicating that these enzymes display both L,D-transpeptidase (LDT) and L,D-  
95 carboxypeptidase (LD-CPase) activities (Fig. 1A and Table 1). The dimer contained a 3→3  
96 cross-link connecting two disaccharide-tripeptides (Table 1). This may imply two mutually  
97 non-exclusive reaction schemes involving both the LDT and LD-CPase activities of  $Ldt_{Cd2}$   
98 and  $Ldt_{Cd3}$ . For the first reaction scheme, the disaccharide-tripeptide generated by the LD-  
99 CPase activity is used as the acyl-acceptor substrate to generate the dimer (as depicted in Fig.  
100 1A). For the second reaction scheme, the cross-linking reaction involves an acyl acceptor  
101 containing a tetrapeptide stem and D-Ala<sup>4</sup> is cleaved off from the dimer by the LD-CPase  
102 activity.

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

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

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

116 Table 1). In *M. tuberculosis*, one of the five L,D-transpeptidase paralogues (Ldt<sub>M15</sub>) similarly  
117 catalyzes formation of peptidoglycan dimers *in vitro* in the absence of detectable acylation by  
118  $\beta$ -lactams (18).

119 For Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub>, mass spectrometry analysis revealed the formation of acyl-  
120 enzymes for representatives of three classes of  $\beta$ -lactams (Table 2). For carbapenems, the  
121 mass of the acyl-enzymes (EI\*) corresponded to the mass of the proteins plus the mass of the  
122  $\beta$ -lactam indicating the presence of the entire drugs in the covalent adducts (Fig. 2). Acylation  
123 of the L,D-transpeptidases by the cephalosporin ceftriaxone led to the loss of the R<sub>2</sub> side-chain  
124 (acyl-enzyme EI\*\*). The acyl-enzyme containing the complete drug (EI\*) was not detected  
125 suggesting a concerted mechanism involving simultaneous acylation and loss of the R<sub>2</sub> side-  
126 chain, as previously described (17, 19). Incubation of LDTs with ampicillin led to the  
127 formation of two acyl-enzymes containing the complete drug (EI\*) or a drug fragment  
128 following cleavage of the C<sup>5</sup>-C<sup>6</sup> bond of the  $\beta$ -lactam ring (EI\*\*). This unexpected cleavage  
129 of a carbon-carbon bond has been previously reported for L,D-transpeptidases (17) and a D,D-  
130 carboxypeptidase (20).

131 **Kinetics of acylation of Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub> by  $\beta$ -lactams.** Stopped-flow  
132 spectrofluorometry was used to compare the efficacy of inactivation of the L,D-  
133 transpeptidases by  $\beta$ -lactams (Table 3 and Fig. 3). The method was developed for Ldt<sub>fm</sub> from  
134 *E. faecium* (21) and applied to the characterization of the L,D-transpeptidases from *M.*  
135 *tuberculosis* (18). The fluorescence kinetics obtained with these enzymes is bi-phasic (see  
136 Fig. 3C for the example of Ldt<sub>fm</sub>). In the first phase, the fluorescence of the Trp residues of  
137 the enzymes is quenched due to reversible formation of an oxyanion. In the second phase, the  
138 fluorescence intensity increases due to the formation of the acyl-enzyme to the detriment of  
139 the oxyanion. The bi-phasic nature of the fluorescence kinetics is accounted for by the large  
140 variations in the fluorescence of the three forms of the enzyme, which is maximum for the apo

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

158 **Kinetics of  $\beta$ -lactam hydrolysis by  $Ldt_{Cd1}$  and  $Ldt_{Cd2}$ .** The turnover for hydrolysis of  
159  $\beta$ -lactams by the L,D-transpeptidases of *C. difficile* (Table 4) showed that the covalent adducts  
160 formed by acylation of these enzymes by carbapenems were stable. Half-lives greater than  
161 150 min were deduced from the observed turnovers for the hydrolysis of the carbapenems,  
162 which were smaller than  $8 \times 10^{-5} \text{ s}^{-1}$  (see reference 17 for the calculation). The acyl-enzyme  
163 formed with ampicillin and  $Ldt_{Cd2}$  was the least stable, as previously described (17), with a  
164 half-life of 2.0 min. This value indicates that  $Ldt_{Cd2}$  is poorly inhibited by ampicillin although  
165 the low turnover for hydrolysis implies that the enzyme is unlikely to contribute to resistance



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

171 **Conclusions.** Peltier *et al.* (9) have previously shown that the peptidoglycan of *C.*  
172 *difficile* contains an unusually high (73%) content of 3 $\rightarrow$ 3 cross-links in comparison to all  
173 other firmicutes that have been submitted to peptidoglycan structure analysis, typically <10%  
174 or 0 % depending upon the presence (*e.g. E. faecium*) or absence (*e.g. Staphylococcus aureus*)  
175 of genes encoding members of the LDT family in their genome (22). Deletion of the genes  
176 encoding Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub>, alone or in combination, was associated with a decrease in the  
177 proportion of mucopeptide dimers containing a 3 $\rightarrow$ 3 cross links from 41% for the wild-type  
178 strain to 19%, 26%, and 13%, respectively (9). This observation provided evidence that Ldt<sub>Cd1</sub>  
179 and Ldt<sub>Cd2</sub> form 3 $\rightarrow$ 3 cross links *in vivo* (9). For an unknown reason, the proportion of the  
180 4 $\rightarrow$ 3 cross links was also reduced, albeit to a lower extent (from 15% for the wild-type strain  
181 to 11%, 11%, and 12% for deletion of genes *ldt*<sub>Cd1</sub>, *ldt*<sub>Cd2</sub>, and *ldt*<sub>Cd1</sub> plus *ldt*<sub>Cd2</sub>, respectively)  
182 (9). Consequently, the deletions of *ldt*<sub>Cd1</sub>, *ldt*<sub>Cd2</sub>, and *ldt*<sub>Cd1</sub> plus *ldt*<sub>Cd2</sub> led to a decrease in the  
183 proportion of dimers (from 57% for the wild-type strain to 30%, 37%, and 34%, respectively),  
184 with a moderate impact on the relative proportions of 3 $\rightarrow$ 3 cross links among dimers, which  
185 were only reduced from 73% for the wild-type strain to 64%, 70% and 66%, respectively (9).  
186 These observations indicate that increased formation of 4 $\rightarrow$ 3 cross links by the PBPs did not  
187 compensate for the absence of Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub>, resulting in a less cross-linked  
188 peptidoglycan.

189 The detection of 3 $\rightarrow$ 3 cross-links in the double mutant lacking Ldt<sub>Cd1</sub> and Ldt<sub>Cd2</sub>  
190 revealed the existence of a third L,D-transpeptidase in *C. difficile* (9). The corresponding gene

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

203

## 204 MATERIALS AND METHODS

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

216           **Production and purification of L,D-transpeptidases.** The vector pET-TEV (our  
217 laboratory collection) is a derivative of pET28a (Novagen) conferring resistance to kanamycin  
218 and enabling to obtain translational fusions consisting of a polyhistidine tag followed by a  
219 TEV protease cleavage site (MHHHHHHENLYFQGHM), as previously used for production  
220 of recombinant L,D-transpeptidases (17). *E. coli* BL21(DE3) harboring the recombinant pET-  
221 TEV (17) derivatives encoding Ldt<sub>Cd1</sub>, Ldt<sub>Cd2</sub>, and Ldt<sub>Cd3</sub> were grown in brain-heart infusion  
222 broth (Difco) containing kanamycin (50 µg/ml) at 37°C until the optical density at 600 nm  
223 reached 0.8. Isopropyl-β-D-1-thiogalactopyranoside (0.5 mM) was added and the incubation  
224 was continued for 18 h at 16°C. Bacteria were collected by centrifugation (5,200 X g; 4°C),  
225 resuspended in 100 mM sodium phosphate buffer (pH 6.4) containing 300 mM NaCl (buffer  
226 A), and lysed by sonication. Cell debris was removed by centrifugation (17,400 X g; 4°C).  
227 The clarified lysate was filtered and LDTs were purified by nickel-affinity chromatography  
228 (Ni-NTA resin, Sigma-Aldrich) and size-exclusion chromatography (Superdex column 75 HL  
229 26/60; GE HealthCare) in buffer A. LDTs were concentrated by ultrafiltration (Amicon Ultra-  
230 4 centrifugal filter devices; Millipore) to a final concentration of 1 mg/ml and stored at -65°C  
231 in buffer A. LDTs were purified in the absence of a reducing agent, as previously described  
232 (*e.g.* ref 18), as the active-site residue of this family of enzymes is not readily oxidized.

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

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

247 **Mass spectrometry analyses of L,D-transpeptidase acylation by  $\beta$ -lactams.** L,D-  
248 transpeptidases (10  $\mu\text{M}$ ) were incubated with  $\beta$ -lactams (100  $\mu\text{M}$ ) in a volume of 5  $\mu\text{l}$  for 60  
249 min at 20°C in 100 mM sodium-phosphate buffer (pH 6.0). The reaction mixture was injected  
250 into the mass spectrometer (Qstar Pulsar I, Applied Biosystem) at a flow rate of 0.05 ml/min  
251 (acetonitrile 50%, water 49.5%, formic acid 0.5 %; per volume). Spectra were acquired in the  
252 positive mode, as previously described (16).

253 **Kinetics of L,D-transpeptidase inactivation by  $\beta$ -lactams.** Fluorescence data were  
254 acquired with a stopped-flow apparatus (RX-2000, Applied Biophysics) coupled to a  
255 spectrofluorometer (Cary Eclipse; Varian) in 100 mM sodium-phosphate (pH 6.0) at 10°C  
256 (17, 21). Hydrolysis of  $\beta$ -lactams by the LDTs was determined by spectrophotometry in  
257 sodium phosphate buffer (100 mM; pH 6.0) at 20°C in a Cary 100 spectrophotometer  
258 (Varian) (17, 21). The variation in the molar extinction coefficient resulting from opening of  
259 the  $\beta$ -lactam ring has been previously determined for carbapenems ( $-7,100 \text{ M}^{-1} \text{ cm}^{-1}$  at 299  
260 nm), ceftriaxone ( $-9,600 \text{ M}^{-1} \text{ cm}^{-1}$  at 265 nm), and ampicillin ( $-700 \text{ M}^{-1} \text{ cm}^{-1}$  at 240 nm) (17).

261

## 262 **ACKNOWLEDGEMENTS**

263 We thank L. Dubost and A. Marie for technical assistance in the collection of mass spectra  
264 (Bio-organic Mass Spectrometry technical platform, Museum National d'Histoire Naturelle).

265

266 **Funding**

267 LS was supported by la Fondation pour la Recherche Médicale (DEA20150633341).

268

269 **Transparency declaration**

270 We have no competing interests to declare.

271

272

273 **REFERENCES**

- 274 1. Barbut F, Petit JC. 2001. Epidemiology of *Clostridium difficile*-associated infections. Clin  
275 Microbiol Infect 7:405-410.
- 276 2. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM,  
277 Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN,  
278 McDonald LC. 2015. Burden of *Clostridium difficile* infection in the United States. N Engl J  
279 Med 372:825-834.
- 280 3. Ananthakrishnan AN. 2011. *Clostridium difficile* infection: epidemiology, risk factors and  
281 management. Nat Rev Gastroenterol Hepatol 8:17-26.
- 282 4. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, Drudy D, Fitzpatrick F,  
283 Wiuff C, Brown DJ, Coia JE, Pituch H, Reichert P, Even J, Mossong J, Widmer AF, Olsen KE,  
284 Allerberger F, Notermans DW, Delmee M, Coignard B, Wilcox M, Patel B, Frei R, Nagy E,  
285 Bouza E, Marin M, Akerlund T, Virolainen-Julkunen A, Lyytikäinen O, Kotila S, Ingebretsen A,  
286 Smyth B, Rooney P, Poxton IR, Monnet DL. 2008. Update of *Clostridium difficile* infection due  
287 to PCR ribotype 027 in Europe, 2008. Euro Surveill 13.
- 288 5. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical M, Infectious D. 2014. European  
289 Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance  
290 document for *Clostridium difficile* infection. Clin Microbiol Infect 20 Suppl 2:1-26.
- 291 6. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, Kuijper EJ,  
292 Wilcox MH. 2008. Emergence of reduced susceptibility to metronidazole in *Clostridium*  
293 *difficile*. J Antimicrob Chemother 62:1046-1052.
- 294 7. Cornely OA, Crook DW, Esposito R, Poirier A, Somero MS, Weiss K, Sears P, Gorbach S, Group  
295 OPTCS. 2012. Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in  
296 Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial.  
297 Lancet Infect Dis 12:281-289.

- 298 8. Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, Gorbach S, Sears P, Shue YK,  
299 Group OPTCS. 2011. Fidaxomicin versus vancomycin for *Clostridium difficile* infection. N Engl  
300 J Med 364:422-431.
- 301 9. Peltier J, Courtin P, El Meouche I, Lemee L, Chapot-Chartier MP, Pons JL. 2011. *Clostridium*  
302 *difficile* has an original peptidoglycan structure with a high level of *N*-acetylglucosamine  
303 deacetylation and mainly 3-3 cross-links. J Biol Chem 286:29053-29062.
- 304 10. Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L,  
305 Mainardi JL. 2008. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis*  
306 predominantly contains cross-links generated by L,D-transpeptidation. J Bacteriol 190:4360-  
307 4366.
- 308 11. Lavollay M, Fourgeaud M, Herrmann JL, Dubost L, Marie A, Gutmann L, Arthur M, Mainardi  
309 JL. 2011. The peptidoglycan of *Mycobacterium abscessus* is predominantly cross-linked by  
310 L,D-transpeptidases. J Bacteriol 193:778-782.
- 311 12. Hugonnet JE, Mengin-Lecreulx D, Monton A, den Blaauwen T, Carbonnelle E, Veckerle C,  
312 Brun YV, van Nieuwenhze M, Bouchier C, Tu K, Rice LB, Arthur M. 2016. Factors essential for  
313 L,D-transpeptidase-mediated peptidoglycan cross-linking and beta-lactam resistance in  
314 *Escherichia coli*. Elife 5.
- 315 13. Biarrotte-Sorin S, Hugonnet JE, Delfosse V, Mainardi JL, Gutmann L, Arthur M, Mayer C. 2006.  
316 Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. J Mol Biol  
317 359:533-538.
- 318 14. Mainardi JL, Morel V, Fourgeaud M, Cremniter J, Blanot D, Legrand R, Frehel C, Arthur M, Van  
319 Heijenoort J, Gutmann L. 2002. Balance between two transpeptidation mechanisms  
320 determines the expression of beta-lactam resistance in *Enterococcus faecium*. J Biol Chem  
321 277:35801-35807.

- 322 15. Mainardi JL, Fourgeaud M, Hugonnet JE, Dubost L, Brouard JP, Ouazzani J, Rice LB, Gutmann  
323 L, Arthur M. 2005. A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant  
324 transpeptidation pathway. *J Biol Chem* 280:38146-38152.
- 325 16. Mainardi JL, Hugonnet JE, Rusconi F, Fourgeaud M, Dubost L, Moumi AN, Delfosse V, Mayer  
326 C, Gutmann L, Rice LB, Arthur M. 2007. Unexpected inhibition of peptidoglycan LD-  
327 transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. *J Biol Chem*  
328 282:30414-30422.
- 329 17. Triboulet S, Dubee V, Lecoq L, Bougault C, Mainardi JL, Rice LB, Etheve-Quellejeu M,  
330 Gutmann L, Marie A, Dubost L, Hugonnet JE, Simorre JP, Arthur M. 2013. Kinetic features of  
331 L,D-transpeptidase inactivation critical for beta-lactam antibacterial activity. *PLoS One*  
332 8:e67831.
- 333 18. Cordillot M, Dubee V, Triboulet S, Dubost L, Marie A, Hugonnet JE, Arthur M, Mainardi JL.  
334 2013. *In vitro* cross-linking of *Mycobacterium tuberculosis* peptidoglycan by L,D-  
335 transpeptidases and inactivation of these enzymes by carbapenems. *Antimicrobial Agents*  
336 *and Chemotherapy* 57:5940-5945.
- 337 19. Dubee V, Triboulet S, Mainardi JL, Etheve-Quellejeu M, Gutmann L, Marie A, Dubost L,  
338 Hugonnet JE, Arthur M. 2012. Inactivation of *Mycobacterium tuberculosis* L,D-transpeptidase  
339 Ldt<sub>Mt1</sub> by carbapenems and cephalosporins. *Antimicrob Agents Chemother* 56:4189-4195.
- 340 20. Marquet A, Frere JM, Ghuysen JM, Loffet A. 1979. Effects of nucleophiles on the breakdown  
341 of the benzylpenicilloyl-enzyme complex EI formed between benzylpenicillin and the  
342 exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* strain R61. *Biochem J*  
343 177:909-916.
- 344 21. Triboulet S, Arthur M, Mainardi JL, Veckerle C, Dubee V, Nguem-Nouri A, Gutmann L, Rice  
345 LB, Hugonnet JE. 2011. Inactivation kinetics of a new target of beta-lactam antibiotics. *J Biol*  
346 *Chem* 286:22777-22784.



- 347 22. Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M. 2008. Evolution of peptidoglycan  
348 biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS  
349 Microbiol Rev 32:386-408.
- 350 23. Arbeloa A, Hugonnet JE, Sentilhes AC, Josseaume N, Dubost L, Monsempes C, Blanot D,  
351 Brouard JP, Arthur M. 2004. Synthesis of mosaic peptidoglycan cross-bridges by hybrid  
352 peptidoglycan assembly pathways in gram-positive bacteria. J Biol Chem 279:41546-41556.
- 353 24. Auger G, van Heijenoort J, Mengin-Lecreulx D, Blanot D. 2003. A MurG assay which utilises a  
354 synthetic analogue of lipid I. FEMS Microbiol Lett 219:115-119.
- 355

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

	Reaction product (calculated mass)		
	Tri <sup>a</sup>	Dimer <sup>a</sup>	Tetra (D-Met) <sup>b</sup>
LDT	(828.36)	(1,638.71)	(959.40)
Ldt <sub>Cd1</sub>	828.37	ND	ND
Ldt <sub>Cd2</sub>	828.36	1,638.71	959.38
Ldt <sub>Cd3</sub>	828.37	1,638.66	959.40

356 <sup>a</sup> LDTs were incubated with the disaccharide-tetrapeptide GlcN-MurNAc-L-Ala- $\gamma$ -D-Glu-  
357 DAP-D-Ala leading to the formation of a disaccharide-tripeptide (L,D-carboxypeptidase  
358 activity) and of a dimer containing a 3 $\rightarrow$ 3 cross-link (L,D-transpeptidase activity).

359 <sup>b</sup> LDTs were incubated with the disaccharide-tetrapeptide and D-Met leading to the exchange  
360 of D-Ala<sup>4</sup> by D-Met.

361 ND, not detected; Tri, GlcN-MurNAc-L-Ala- $\gamma$ -D-Glu-DAP; Tetra(D-Met), GlcN-MurNAc-L-  
362 Ala- $\gamma$ -D-Glu-DAP-D-Met.

363

**TABLE 2** Mass of L,D-transpeptidases (LDTs) and of acyl-enzymes formed with various  $\beta$ -lactams

LDT		Enzyme or acyl-enzyme		Mass	
$\beta$ -lactam	(Mass)	(expected mass increase)		Calculated	Observed
Ldt <sub>Cd1</sub>		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 <sub>Cd2</sub>		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

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

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

$\beta$ -lactam	Acylation efficacy ( $M^{-1} s^{-1}$ ) <sup>a</sup>	
	Ldt <sub>Cd1</sub>	Ldt <sub>Cd2</sub>
Ampicillin	3.9 $\pm$ 0.6	66 $\pm$ 3
Ceftriaxone	82 $\pm$ 20	40 $\pm$ 1
Imipenem	3,100 $\pm$ 400	6,600 $\pm$ 100
Meropenem	1,100 $\pm$ 400	480 $\pm$ 20
Ertapenem	3,200 $\pm$ 500	1,030 $\pm$ 20

370 <sup>a</sup> Values are the mean  $\pm$  standard error from the linear regression for the kinetic parameter

371  $k_2/K_{app}$ .

372 <sup>b</sup> ~~The acylation of the LDTs was not detected by spectrofluorometry. ND, not detected.~~

373

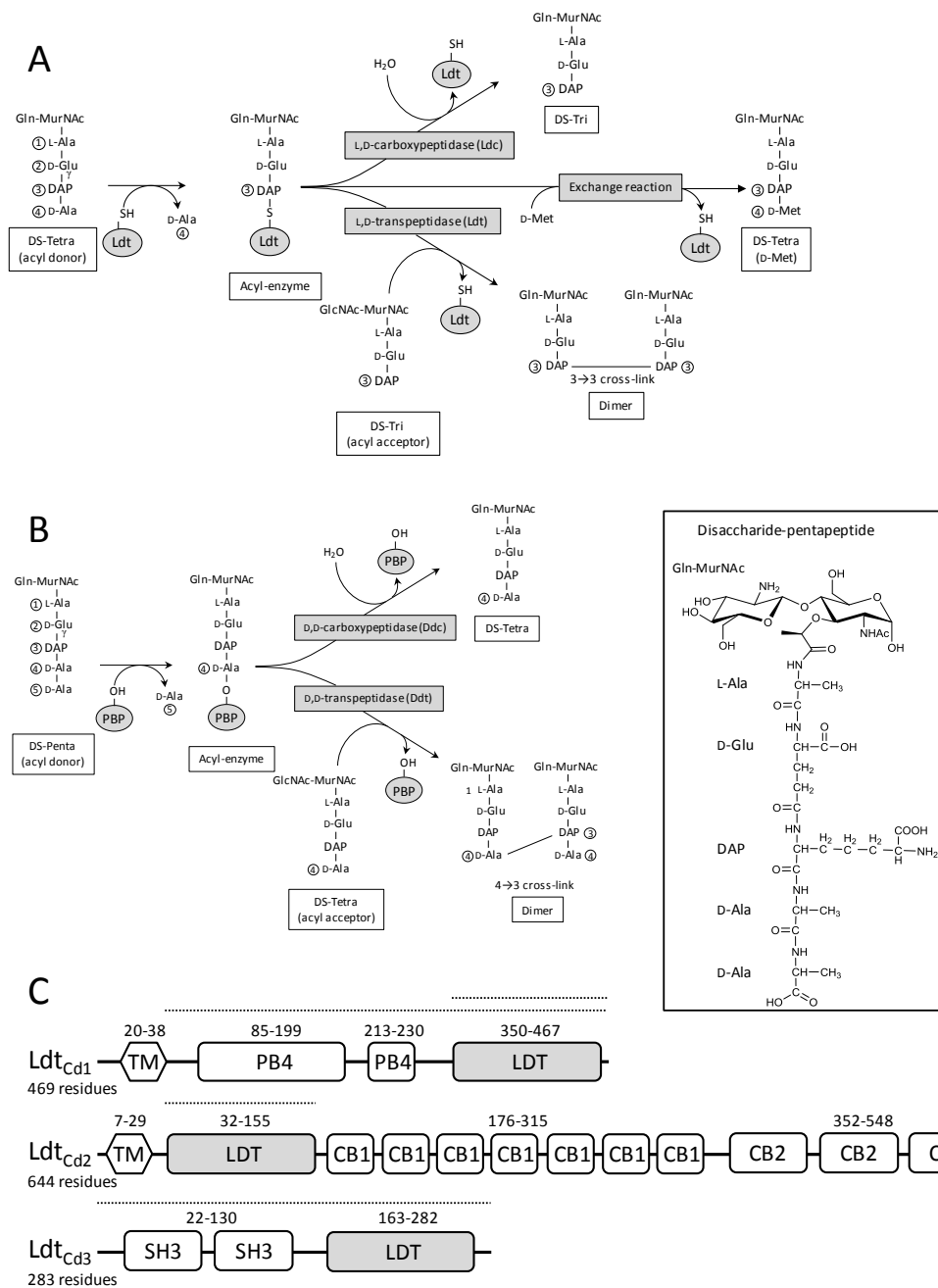
374

375

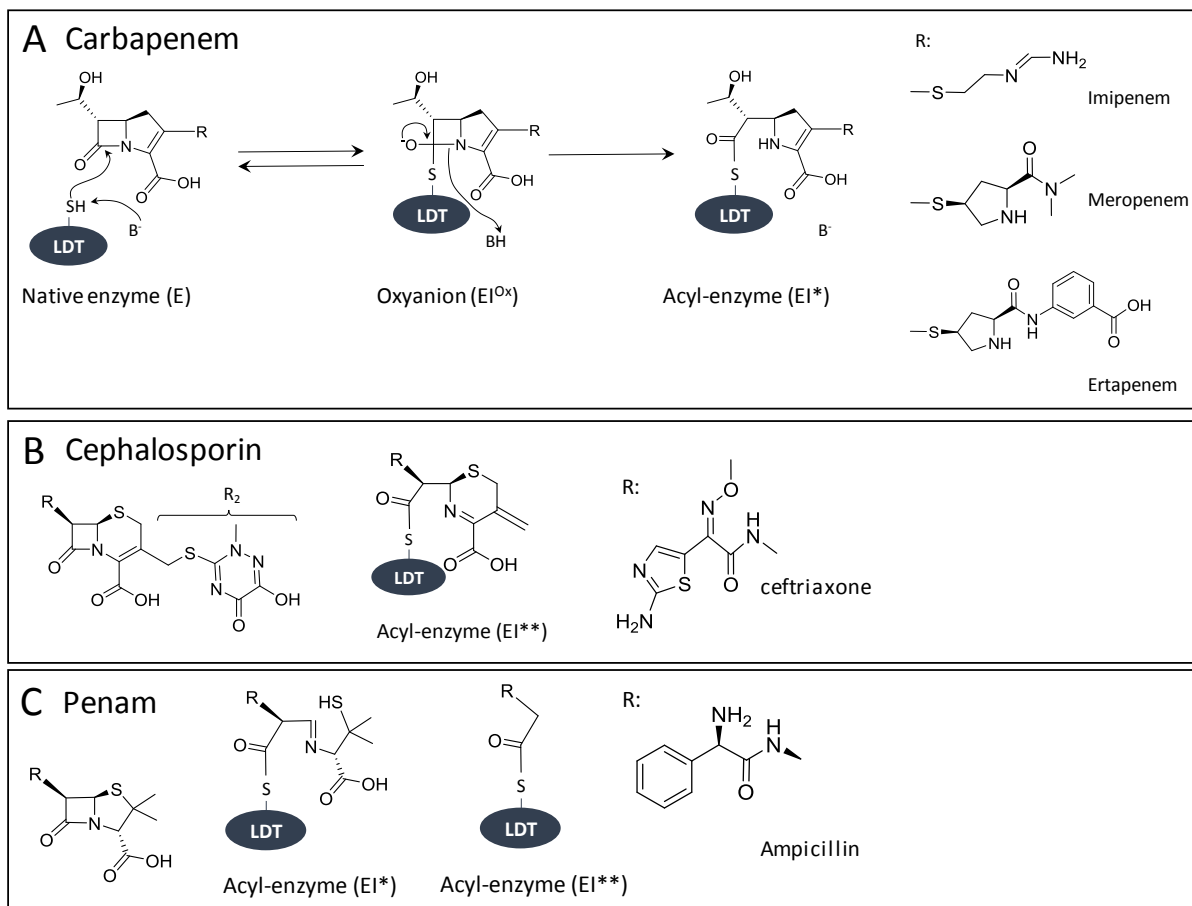
**TABLE 4** Hydrolysis of  $\beta$ -lactams by *C. difficile* L,D-transpeptidases

$\beta$ -lactam	Hydrolysis turnover $\times 10^6$ ( $s^{-1}$ ) <sup>a</sup>	
	Ldt <sub>Cd1</sub>	Ldt <sub>Cd2</sub>
Ampicillin	< 1,000	5,800 $\pm$ 400
Ceftriaxone	160 $\pm$ 10	180 $\pm$ 15
Imipenem	< 180	< 180
Meropenem	30 $\pm$ 5	48 $\pm$ 7
Ertapenem	40 $\pm$ 3	79 $\pm$ 13

376 <sup>a</sup> Values are the mean  $\pm$  standard error from the linear regression for the turnover

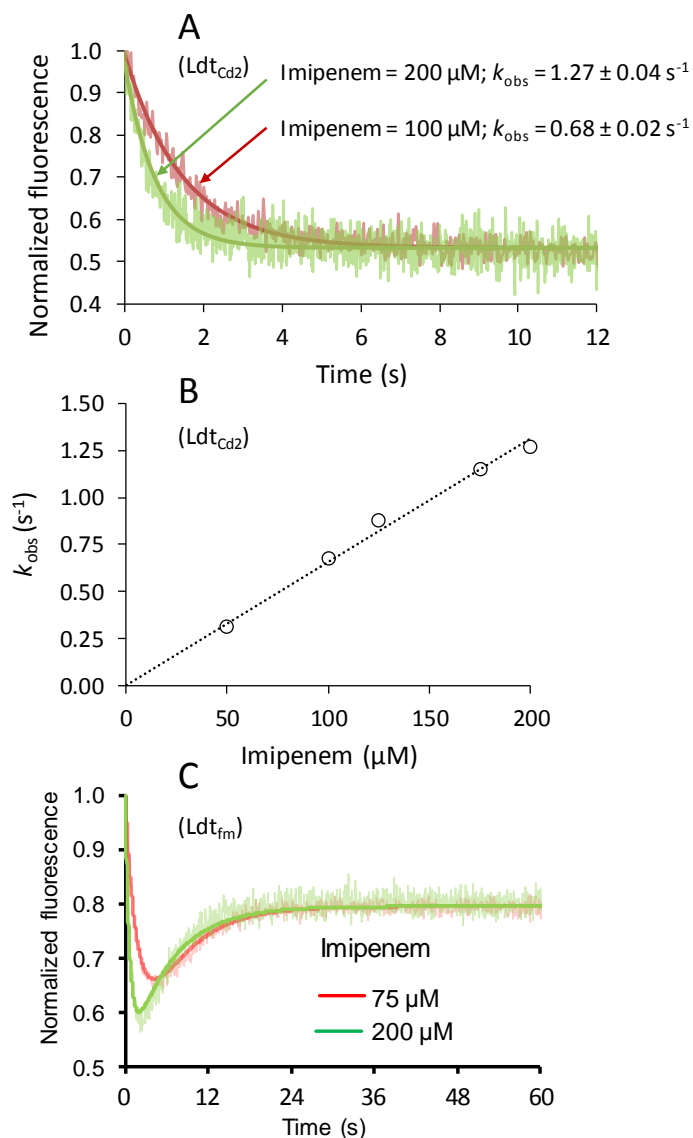


377  
 378 **FIG 1** Peptidoglycan cross-linking in *C. difficile*. The inset shows the developed structure of  
 379 the disaccharide-pentapeptide subunit of *C. difficile*. (A) Reactions catalyzed by L,D-  
 380 transpeptidases (Ldt). (B) Reactions catalyzed by penicillin-binding proteins (PBPs). (C)  
 381 Domain composition of Ldt<sub>Cd1</sub>, Ldt<sub>Cd2</sub>, and Ldt<sub>Cd3</sub>. Numbers refer to the boundaries of the  
 382 domains. Dashed lines indicate the portions of Ldt<sub>Cd1</sub> (residues 39-469 and 350-469), Ldt<sub>Cd2</sub>  
 383 (30-156), and Ldt<sub>Cd3</sub> (1-256) present in recombinant proteins produced in *E. coli*. These  
 384 portions of the proteins comprise (i) the entire protein without the putative transmembrane  
 385 segment or the catalytic L,D-transpeptidase domain for Ldt<sub>Cd1</sub>, (ii) the catalytic domain for  
 386 Ldt<sub>Cd2</sub>, and (iii) the entire protein for Ldt<sub>Cd3</sub>. The recombinant proteins contained an additional  
 387 vector-encoded N-terminal 6 x His Tag followed by a TEV cleavage site  
 388 (MGSSHHHHHSSGENLYFQGHM). Domain identification is based on sequences  
 389 similarities in the absence of functional data. CB1 and CB2, cell wall binding type 1 and 2  
 390 domains, respectively; Ldt, catalytic L,D-transpeptidase domain; PB4, protein-binding pattern  
 391 type 4; SH3, bacterial domain SH3; TM, transmembrane segment.



392  
393  
394  
395  
396

**FIG 2** Acyl-enzymes formed by *C. difficile* L,D-transpeptidases with  $\beta$ -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<sub>Cd2</sub> by imipenem. (A)  
 398 Examples of stopped-flow fluorescence kinetics obtained with Ldt<sub>Cd2</sub> (10 μM) and two  
 399 concentrations of imipenem (100 and 200 μM). The rate constant  $k_{\text{obs}}$  was determined by  
 400 fitting data to exponential decay. (B) The rate constant  $k_{\text{obs}}$  increased linearly with the  
 401 concentration of imipenem. The slope provided an estimate of the efficacy of enzyme  
 402 acylation ( $6,600 \pm 100 \text{ M}^{-1} \text{ s}^{-1}$ ). (C) Examples of stopped-flow fluorescence kinetics obtained  
 403 with Ldt<sub>fm</sub> (10 μM) and two concentrations of imipenem (from reference 17).