Tryptophan fluorescence quenching in β -lactam-interacting proteins is modulated by the structure of intermediates and final products of the acylation reaction

Sébastien Triboulet^{a1}, Zainab Edoo^{a1}, Fabrice Compain^{a,b}, Clément Ourghanlian^a, Adrian Dupuis^a, Vincent Dubée^a, Laetitia Sutterlin^a, Heiner Atze^a, Mélanie Ethève-Quelquejeu^c, Jean-Emmanuel Hugonnet^{a2}, and Michel Arthur^{a2}

¹Equal contribution

²Equal contribution and corresponding authors

[a] Dr. S Triboulet, Ms Z Edoo, Dr. F Compain, Dr. C Ourghanlian, Mr A Dupuis, Dr. V Dubée, Dr. L Sutterlin, Dr. H Atze, Dr. JE Hugonnet, Dr. M Arthur, Sorbonne Université, Sorbonne Paris Cité, Université Paris Descartes, Université Paris Diderot, INSERM, Centre de Recherche des Cordeliers, CRC, F-75006 Paris, France. E-mail: jean-emmanuel.hugonnet@crc.jussieu.fr; michel.arthur@crc.jussieu.fr

[b] Dr. F Compain, Service de Microbiologie, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France.

[c] Dr. M Ethève-Quelquejeu, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, Université Paris Descartes, UMR 8601, Paris, F-75005 France; CNRS UMR 8601, Paris, F-75006 France.

Keywords: acylenzyme, β -lactamase, fluorescence quenching, L,D-transpeptidase, tryptophan fluorescence.

In most bacteria, β -lactam antibiotics inhibit the last cross-linking step of peptidoglycan synthesis by acylation of the active-site Ser of D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family. In mycobacteria, cross-linking is mainly ensured by L,D-transpeptidases (LDTs), which are promising targets for the development of β -lactam-based therapies for multidrug-resistant tuberculosis. For this purpose, fluorescence spectroscopy is used to investigate the efficacy of LDT inactivation by β -lactams but the basis for fluorescence quenching during enzyme acylation remains unknown. In contrast to what has been reported for PBPs, we show here using a model L,D-transpeptidase (Ldt_{fm}) that fluorescence quenching of Trp residues does not depend upon direct hydrophobic interaction between Trp residues and β -lactams. Rather, Trp fluorescence was quenched by the drug covalently bound to the active-site Cys residue of Ldt_{fm}. Fluorescence quenching was not quantitatively determined by the size of the drug and was not specific of the thioester link connecting the β -lactam carbonyl to the catalytic Cys as quenching was also observed for acylation of the active-site Ser of β -lactamase BlaC from *M. tuberculosis*. Fluorescence quenching was extensive for reaction intermediates containing an amine anion and for acylenzymes containing an imine stabilized by mesomeric effect, but not for acylenzymes containing a protonated β -lactam nitrogen. Together, these results indicate that the extent of fluorescence quenching is determined by the status of the β -lactam nitrogen. Thus, fluorescence kinetics can provide information not only on the efficacy of enzyme inactivation but also on the structure of the covalent adducts responsible for enzyme inactivation.

Peptidoglycan is an essential constituent of bacterial cell walls since it prevents cell swelling and lysis by mechanically sustaining the osmotic pressure of the cytoplasm¹. This protective function depends upon synthesis and maintenance during the entire cell cycle of the net-like peptidoglycan macromolecule, which completely surrounds the bacterial cell. Peptidoglycan is made of glycan strands cross-linked by short peptide stems. In most bacteria, the cross-linking step is performed by D,D-transpeptidases, which are the essential targets of β -lactam antibiotics and are often referred to as penicillin-binding proteins (PBPs)². In mycobacteria³⁻⁴ and in *Clostridium difficile*⁵⁻⁶, the cross-links found are mainly (70% to 80%) formed by a second class of enzymes, the L,D-transpeptidases (LDTs). Since LDTs are not inhibited by β -lactams belonging to the penam class, such as ampicillin,⁷⁻⁸ these enzymes are responsible for high-level resistance to these drugs in mutants of *Enterococcus faecium*⁹⁻¹⁰ and *Escherichia coli*¹¹ selected in laboratory conditions.

PBPs and LDTs are structurally unrelated¹²⁻¹⁵ and proceed through different catalytic mechanisms for activation of Ser and Cys nucleophiles¹⁶, which are part of Lys-Ser¹⁷ and Cys-His-Asp¹⁸ catalytic diad and triad, respectively. PBPs and LDTs also differ by the structure of the stem peptide used as an acyl donor, a pentapeptide for PBPs¹⁷ and a tetrapeptide for LDTs¹⁶, except for *Enterococcus faecalis* Ldt_{fs}¹⁹. PBPs cleave the D-Ala⁴-D-Ala⁵ peptide bond of the acyl donor, hence the D,D designation for these transpeptidases, and link the carbonyl of D-Ala⁴ to the side-chain amino group of the 3rd residue thereby generating 4→3 cross-links¹⁷ (Supplementary Fig. S1). In contrast, LDTs cleave the L-Lys³-D-Ala⁴ bond of the donor (L,D designation) and form 3→3 cross-links¹⁶.

The first substrate of the transpeptidation reaction, the acyl donor, forms an acylenzyme intermediate that subsequently reacts with the second substrate, the acceptor, leading to the final cross-linked product. For PBPs, nucleophilic attack of the carbonyl of D-Ala⁴ by the active-site Ser results in an acylenzyme containing an ester bond and release of D-Ala⁵¹⁷. For the acylenzyme formed by LDTs, a thioester bond connects the carbonyl of L-Lys³ to the γ sulfur of the active-site Cys¹⁶. β -

lactams are structure analogues of the D-Ala⁴-D-Ala⁵ extremity of stem pentapeptides and act as suicide substrates of the PBPs²⁰. Nucleophilic attack of the carbonyl of the β -lactam ring by the active-site Ser of PBPs leads to formation of an acylenzyme, which is only hydrolyzed very slowly, leading in practice to “irreversible” inactivation in the time scale of a bacterial generation (Supplementary Fig. S1)². LDTs are also acylated by β -lactams although efficacious enzyme inactivation and antibacterial activity occur only for a single class of β -lactams, the carbapenems, such as imipenem⁷⁻⁸. β -lactams of the cephem (cephalosporin) class, such as ceftriaxone, are only active at high concentrations since acylation is slower and the resulting thioester bond is prone to hydrolysis⁷. The kinetic parameters are even less favorable for penams (ampicillin and penicillin) leading to equilibrium between the acylated and unacylated (functional) forms, which accounts for the lack of antibacterial activity⁷.

Fluorescence kinetics revealed that the acylation reaction of LDTs by β -lactams of the carbapenem class comprises two limiting steps (Fig. 1A and B). In the first step, nucleophilic attack of the β -lactam carbonyl by the negatively charged sulfur atom of the catalytic Cys was thought to lead to formation of a covalent sulfur-carbon bond and to the development of a negative charge on the drug²¹. Initially, this negative charge was proposed to be located on the β -lactam oxygen²¹. This hypothesis was based on analogies with PBPs, which contain an oxyanion hole for stabilization of the negative charge developing on the β -lactam oxygen¹⁷. However, hybrid potential simulation of the acylation reaction indicated that the path to an oxyanion is energetically disfavored in the case of LDTs and that an oxyanion could not correspond to a stabilized reaction intermediate²². Rather, hybrid potential simulation indicated that the most energetically favored reaction path involves a concerted mechanism leading to the concomitant formation of the thioester bond, rupture of C-N β -lactam bond, and formation of an amine anion²². In the second step, the final acylenzyme is formed by protonation of the amine anion. The origin of this proton remains elusive^{18,22}. Formation of a non-covalent complex is not considered in this reaction scheme as the affinity of the Ldt_{fm} for carbapenems is very low, in the order of 60 to 80 mM⁷.

Fluorescence kinetics proved useful to assess the efficacy of inactivation of LDTs from *Mycobacterium tuberculosis*²³⁻²⁵ and contributed to the choice of the drugs that were tested in a phase II clinical trial²⁶. The assays were also useful in evaluating series of synthetic carbapenems²⁷ and non- β -lactam LDT inhibitors²⁸. However, the basis for the variation in fluorescence intensity detected during the two steps of the acylation reaction remained elusive. Initially, we developed fluorescence kinetics since determination of the crystal structure of Ldt_{fm} revealed a Trp residue at the entrance of the catalytic cavity¹⁵, which appeared ideally located for fluorescence quenching upon drug binding due to changes in its environment²¹. However, this naïve explanation was subsequently found to be insufficient to account for the various behaviors observed for acylation of LDTs by representatives of the β -lactam classes^{7,22-25}. This prompted us to investigate here the basis for variations in fluorescence intensity occurring upon acylation of LDTs using the well-characterized L,D-transpeptidase Ldt_{fm} from *E. faecium* as a model. We show that variations in fluorescence intensity are not due to modification of the environment of Trp residues but to the formation of a β -lactam-derived fluorescence quencher during the acylation reaction.

RESULTS

Trp to Phe and His to Ala substitutions do not abolish the biphasic behavior of fluorescence kinetics observed for Ldt_{fm} inactivation by imipenem. We have previously proposed that the acylation of Ldt_{fm} by carbapenems is a two-step reaction involving reversible formation of an amine anion (EI^{An}, step 1)

followed by irreversible formation of an acylenzyme (EI^* , step 2) (Fig. 1A) ²¹⁻²². Fluorescence kinetics also display two phases enabling the determination of kinetic parameters for the two steps of the reaction (Fig. 1B) ²¹. In the first phase, fluorescence intensity decreases because EI^{An} is rapidly formed to the detriment of the free enzyme (E) and the fluorescence intensity of EI^{An} is lower than that of E . In the second phase, fluorescence intensity increases since the acylenzyme (EI^*) is formed to the detriment of EI^{An} (acylation step) and the fluorescence intensity of EI^* is greater than that of EI^{An} . Our first aim was to determine whether variations in the fluorescence intensity could be correlated to the modification of the environment of a specific Trp residue of Ldt_{fm} . To address this question, Trp residues of the catalytic domain of Ldt_{fm} were replaced by Phe residues in various combinations. For the sake of simplicity, the six Trp residues at positions 355, 385, 410, 415, 425, and 434 of Ldt_{fm} were designated as residues a to f, respectively. Fluorescence kinetics were determined for Ldt_{fm} derivatives lacking 2 to 5 Trp residues (Fig. 1C). Fluorescence kinetics remained bi-phasic in all cases, except for the Ldt_{fm} derivative only retaining the “a” residue (*i.e.* Trp³⁵⁵). Thus, the variations in the fluorescence intensity associated with the two steps of the acylation reaction do not depend upon the modification of the environment of a specific Trp residue of Ldt_{fm} . This prompted us to investigate fluorescence quenching by His residues of Ldt_{fm} . Fluorescence kinetics obtained with the His³⁵³Ala and His⁴⁴⁰Ala derivatives of Ldt_{fm} indicated that neither residue is essential for biphasic fluorescence kinetics (Fig. 1C). His at position 421 could not be analyzed since this residue is part of the catalytic triad and is essential for Ldt_{fm} activity.

Impact of Trp to Phe and His to Ala substitutions on the efficacy of the inactivation reaction and on the relative fluorescence intensity of the three enzyme forms. Fitting simulations to experimental data for four concentrations of imipenem was used to determine the inactivation kinetic parameters (Supplementary Fig. S2). None of the Trp residues was essential for the acylation of Ldt_{fm} by imipenem although important variations were observed in the k_1 (formation of the amine anion) and k_2 (acylation step) parameters (Fig. 2A and 2B, respectively). The Trp to Phe substitutions produced large variations in the intrinsic fluorescence of the three forms of the enzyme (apo Ldt_{fm} , amine anion, and acylenzyme; Fig. 2C). The formation of the acylenzyme (EI^*) was confirmed by mass spectrometry for all Ldt_{fm} derivatives (mass increment of 299.3 Da; data not shown).

Conservation of Trp residues in Ldt_{fm} homologues. The Trp residues were poorly conserved in L,D -transpeptidases from Gram-positive and Gram-negative bacteria (Supplementary Fig. S3), as expected from the substantial high residual acylation activity of Ldt_{fm} derivatives containing Trp to Phe substitutions (above, Fig. 2A and B). However, residue e (Trp⁴²⁵) was relatively conserved in L,D -transpeptidases from *M. tuberculosis*, being present in the Mt1, Mt2, Mt3, and Mt4 enzymes as well as in the corresponding orthologues of *M. abscessus*. These enzymes are known to display bi-phasic fluorescence kinetics upon inactivation by carbapenems ^{23-24, 29} and (unpublished results). Since Trp⁴²⁵ was the only conserved Trp residue in the LDTs from mycobacteria we investigated the impact of a Trp to Phe substitution at this position in Ldt_{fm} . As shown in Supplementary Fig. S4A and B, derivatives of Ldt_{fm} lacking all Trp residues except Trp⁴²⁵ or lacking only this residue displayed biphasic kinetics. These results indicated that the biphasic nature of fluorescence kinetics does not depend upon any conserved Trp residue in the L,D -transpeptidase protein family.

Fluorescence kinetics with a single ectopic Trp residue at position 383. All six Trp residues of Ldt_{fm} were replaced by Phe residues. As expected, the variant devoid of Trp residues was very weakly fluorescent (data not shown). No modification of this weak fluorescence was observed upon acylation

by imipenem. This observation ruled out the formal possibility that the catalytic Cys acylated by imipenem could be a fluorophore. Replacement of Tyr at position 383 by a Trp residue in this Ldt_{fm} derivative restored biphasic fluorescence kinetics (Supplementary Fig. S4C). This result confirmed that biphasic fluorescence kinetics are not dependent upon the presence of any of the six Trp residues of Ldt_{fm} in their original positions.

Investigation of the opened β -lactam ring linked to the catalytic Cys residue as a quencher. Since the variations in the fluorescence observed during the two steps of the acylation reaction could not be accounted for by modification of the environment of any specific Trp residue, our next objective was to investigate whether quenching could result from energy transfer from Trp residues to the Cys- β -lactam covalent adduct generated upon enzyme inactivation. To investigate this possibility, we first examined acylation of Ldt_{fm} by the chromogenic cephalosporin nitrocefirin. Fluorescence kinetics with this compound displayed a monophasic behavior with a large decrease (72%) in the fluorescence intensity (Fig. 3A). In the Ldt_{fm}-nitrocefirin acylenzyme, the negative charge developing on the β -lactam nitrogen is stabilized by a mesomeric effect that prevents protonation of the amine³⁰. Thus, the amine anion formed as an end product with nitrocefirin (Fig. 3A) or as an intermediate with imipenem (Fig. 1) was associated with extensive fluorescence quenching. As found for nitrocefirin, acylation of Ldt_{fm} by ceftriaxone led to a monophasic decrease in the fluorescence intensity (Fig. 3B). Acylation of Ldt_{fm} by ceftriaxone involves a concerted mechanism leading to the elimination of the side chain and formation of a conjugated imine⁷. The extent of fluorescence quenching observed for the acylenzyme formed with ceftriaxone (40%; imine) or by the intermediate formed with imipenem (45%; amine anion) were more important than that observed for the final Ldt_{fm}-imipenem adduct (20%) suggesting that quenching was decreased by protonation of the β -lactam nitrogen.

Fluorescence quenching associated with inactivation of β -lactamases by β -lactamase inhibitors. In order to enrich the structural diversity of molecules investigated in the fluorescence quenching assay, we aimed to test β -lactamase inhibitors. Most β -lactams could not be tested with β -lactamases since the fluorescence assay requires relatively high enzyme concentrations (typically 10 μ M) leading to rapid hydrolysis of the drugs even if the turnovers are low. For this reason, we focused on inactivation of BlaC from *Mycobacterium tuberculosis* by clavulanate since the corresponding acylenzyme is not prone to hydrolysis at the timescale of our experiments³¹. Acylation of BlaC by clavulanate led to a large decrease in the fluorescence intensity (37%) (Fig. 3C). Previous studies have shown that acylation of BlaC by clavulanate leads to formation of imine and *trans*-enamine tautomers (depicted in Fig. 3C) following drug decarboxylation³². Since Ldt_{fm} and BlaC contain Cys and Ser as the active-site nucleophile, respectively, these results indicate that fluorescence quenching is observed with acylenzymes containing both thioester and ester bonds.

Avibactam offered the possibility to test a non- β -lactam inhibitor that inhibits BlaC by formation of a carbamoylenzyme³³. Formation of the adduct (Supplementary Fig. S5) was not associated with any significant modification of the fluorescence intensity of the enzyme (Fig. 3D). Protonation of the amino sulfate nitrogen of avibactam in the avibactam-BlaC adduct was thus associated with limited quenching as found for the protonation of the β -lactam nitrogen in the final Ldt_{fm}-imipenem and BlaC-clavulanate adducts (Fig. 1 and 3C).

Acylation of Ldt_{fm} and BlaC by faropenem. Faropenem, a β -lactam belonging to the penem class, is poorly hydrolyzed by BlaC and rapidly inactivates L,D-transpeptidases²⁴. Thus, faropenem provided the opportunity to study the acylation of the two enzyme types, *i.e.* a β -lactamase (BlaC) and an L,D-

transpeptidase (Ldt_{fm}), by the same β -lactam. Acylation of BlaC by faropenem led to extensive fluorescence quenching (44%) (Fig. 3E). The resulting acylenzyme contained an unprotonated β -lactam nitrogen. In contrast, acylation of Ldt_{fm} by faropenem, which resulted in the rupture of the C⁵-C⁶ bond and the loss of a large portion of the drug including the β -lactam nitrogen²⁴, was associated with a moderate decrease in the fluorescence intensity (17%) (Fig. 3F).

Acylation of BlaC by the meropenem carbapenem. Although BlaC displays moderate carbapenemase activity, previous studies have shown that a BlaC-meropenem adduct is the predominant enzyme form upon incubation of the enzyme with low drug concentrations³⁴⁻³⁵. As expected, the control experiment depicted in Supplementary Fig. S6 shows that the concentration of meropenem remains saturating for at least 80 min during hydrolysis of meropenem (40 – 200 μ M) by BlaC (10 μ M). Fluorescence kinetics at a shorter time scale (6 min) showed a transitory decrease in fluorescence intensity (40%) (Fig. 3G). This observation suggests that formation of an amine anion initially leads to an important fluorescence quenching. Then, fluorescence returned to the initial level suggesting that protonation of the amine anion fully suppressed quenching. Thus, formation of an amine anion following nucleophilic attack of the carbonyl carbon of carbapenems by Ldt_{fm} (Fig. 1) and BlaC (Fig. 3G) similarly led to extensive fluorescence quenching despite the presence of different catalytic residues (Cys *versus* Ser) and different modes of activation of these nucleophiles involving His and Lys residues.

DISCUSSION

The main aim of the present study was to determine the basis for the variations in the fluorescence intensity observed upon acylation of LDTs by β -lactam antibiotics. Previous analyses of the D,D-transpeptidase R61 (PBP family) have shown that acylation of the enzyme by penicillin G leads to a fluorescence quenching, which specifically depends upon one of two Trp residues of the protein³⁶⁻³⁷. This residue, W²³³, is essential for the D,D-carboxypeptidase activity of the protein, as determined by the release of the terminal D-Ala residue from a model peptide ending in D-Ala-D-Ala³⁷. The crystal structure of R61 in complex with a peptide mimicking the acyl donor revealed hydrophobic interactions between the side-chain of W²³³ and methylene groups of this substrate³⁸. Likewise, the structure of a penicillin-R61 complex revealed that W²³³ is in hydrophobic interaction with the phenylacetyl side chain of the drug³⁹ indicating that fluorescence quenching may depend upon this direct interaction. In contrast, we show here that the variations in fluorescence during acylation of LDTs by β -lactam antibiotics require at least one Trp residue but that the position of this residue is not determinant. This was established by deleting Trp residues from Ldt_{fm} in various combinations (Fig. 1 and 2), by introducing a single Trp residue in an ectopic position of Ldt_{fm} (Fig. S4), and by showing that large variations in fluorescence intensity occur in distantly related LDTs and unrelated β -lactamases that do not share any conserved Trp residue (Fig. S3). Since fluorescence quenching was not mainly due to changes in the environment of Trp residues, we investigated the possibility that the nucleophilic attack of the β -lactam ring could itself generate a fluorescence quencher. If this is the case, the fluorescence profile should depend upon the structural features of the drug bound to the active-site residue. Accordingly, extensive quenching was not mainly determined by the size of the side chains of β -lactams or by the nature of the enzyme nucleophile (Cys *versus* Ser in Ldt_{fm} and BlaC, respectively) (Fig. 3). Acylation of Ser or Cys *per se* was not sufficient for fluorescence quenching since a modest or no decrease in fluorescence intensity was observed for the final acylation products of Ldt_{fm} by faropenem, of BlaC by avibactam, and of both enzymes by carbapenems. By elimination, these results pointed to

the status of the β -lactam nitrogen as the only source of variation that could account for the main variations in fluorescence intensity. A large fluorescence quenching occurred if the β -lactam nitrogen was negatively charged, as transiently found during acylation of Ldt_{fm} and BlaC by carbapenems. A large fluorescence quenching also occurred if the nitrogen atom was engaged in a double bond as observed for the acylenzymes formed by Ldt_{fm} with ceftriaxone or nitrocefirin and by BlaC with faropenem or clavulanate. In agreement with the critical role of the double bond, fluorescence quenching was not observed for acylation of BlaC by avibactam, which results in the formation of a carbamoyl-enzyme with a protonated nitrogen, or for acylation of Ldt_{fm} by faropenem, which results in the formation of an acylenzyme lacking the nitrogen atom following rupture of the C⁵-C⁶ bond. These results indicate that fluorescence kinetics can provide information not only on the efficacy of enzyme inactivation but also on the structure of the covalent adducts, including for example the presence of Δ 1- or Δ 2-pyrroline ring in enzyme-carbapenem adducts. Our results also indicate that nitrocefirin provides a very sensitive assay to titrate the active site of enzymes that do not hydrolyze this cephalosporin. This property could be exploited to identify β -lactams and non- β -lactam inhibitors that reversibly or irreversibly bind to LDTs.

EXPERIMENTAL SECTION

Enzyme production and purification. The catalytic domain of Ldt_{fm} (residues 341 to 466) and soluble BlaC (residues 39 to 306) were produced in *E. coli* and purified by metal affinity and size exclusion chromatography as previously described³⁰. Synthetic genes were purchased for production of Ldt_{fm} derivatives with Trp to Phe and His to Ala substitutions (GeneCust). All experiments were performed with a fixed concentration of Ldt_{fm} and BlaC (10 μ M).

Spectrofluorimetry. Fluorescence kinetics were studied in 100 mM sodium phosphate (pH 6.0) at 20°C by using a stopped-flow apparatus (RX-2000, Applied Biophysics) coupled to a spectrofluorimeter (Cary Eclipse; Varian). Excitation was performed at 224 nm with a 5 nm slit and a 2 mm optical path. Fluorescence emission was determined at 335 nm with a 5 nm slit and a 10 mm optical path. Fluorescence data were collected exactly in the same conditions for comparison of the relative fluorescence of wild-type Ldt_{fm} and derivatives with Trp to Phe and His to Ala substitutions. These conditions included the photomultiplier voltage, which was set to 600 V. Kinetic constants were determined using the DynaFit software (BioKin Ltd)⁴⁰ for each Ldt_{fm} derivative by simultaneously fitting the fluorescence data for the various inhibitor concentrations to differential equations derived from the reaction scheme depicted in Fig. 1A.

Mass spectrometry. Formation of the proposed Ldt_{fm}- β -lactam and BlaC- β -lactam adducts in the conditions described in the text was systematically checked by mass spectrometry (data not shown). Enzymes and β -lactams were incubated for appropriate time periods at 20°C. Five μ l of acetonitrile and 1 μ l of 1% formic acid were extemporaneously added. Injection in the mass spectrometer (Qstar Pulsar I; Applied Biosystem) was performed at a flow rate of 0.05 ml/min (acetonitrile, 50%, water, 49.5%, and formic acid, 0.5%; per volume). Spectra were acquired in the positive mode, as previously described⁸. Detailed analyses of the mass spectra of the β -lactam adducts considered in the current study have been previously published^{7-8, 24, 29, 41}.

ANCILLARY INFORMATION

Supporting Information. Supplementary figures S1 to S6.

Corresponding Author Information. Correspondence should be addressed to jean-emmanuel.hugonnet@crc.jussieu.fr and/or michel.arthur@crc.jussieu.fr

Author Contribution. ST, ZE contributed equally to this work. MA and JEH contributed equally to this work.

Acknowledgement. We thank L. Dubost and A. Marie for technical assistance in the collection of mass spectra. This work was supported by the French National Research Agency (grant MycWall, ANR-17-CE18-0010) and the Fondation pour la Recherche Médicale (grant ECO20160736080 to ZE).

REFERENCES

1. Turner, R. D.; Vollmer, W.; Foster, S. J., Different walls for rods and balls: the diversity of peptidoglycan. *Mol Microbiol* **2014**, *91* (5), 862-74.
2. Zapun, A.; Contreras-Martel, C.; Vernet, T., Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol Rev* **2008**, *32* (2), 361-85.
3. Lavollay, M.; Arthur, M.; Fourgeaud, M.; Dubost, L.; Marie, A.; Veziris, N.; Blanot, D.; Gutmann, L.; Mainardi, J. L., The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol* **2008**, *190* (12), 4360-6.
4. Lavollay, M.; Fourgeaud, M.; Herrmann, J. L.; Dubost, L.; Marie, A.; Gutmann, L.; Arthur, M.; Mainardi, J. L., The peptidoglycan of *Mycobacterium abscessus* is predominantly cross-linked by L,D-transpeptidases. *J Bacteriol* **2011**, *193* (3), 778-82.
5. Peltier, J.; Courtin, P.; El Meouche, I.; Lemee, L.; Chapot-Chartier, M. P.; Pons, J. L., *Clostridium difficile* has an original peptidoglycan structure with a high level of N-acetylglucosamine deacetylation and mainly 3-3 cross-links. *J Biol Chem* **2011**, *286* (33), 29053-62.
6. Sutterlin, L.; Edoo, Z.; Hugonnet, J. E.; Mainardi, J. L.; Arthur, M., Peptidoglycan Cross-Linking Activity of L,D-Transpeptidases from *Clostridium difficile* and Inactivation of These Enzymes by beta-Lactams. *Antimicrob Agents Chemother* **2018**, *62* (1).
7. Triboulet, S.; Dubee, V.; Lecoq, L.; Bougault, C.; Mainardi, J. L.; Rice, L. B.; Etheve-Quelquejeu, M.; Gutmann, L.; Marie, A.; Dubost, L.; Hugonnet, J. E.; Simorre, J. P.; Arthur, M., Kinetic features of L,D-transpeptidase inactivation critical for beta-lactam antibacterial activity. *PLoS One* **2013**, *8* (7), e67831.
8. Mainardi, J. L.; Hugonnet, J. E.; Rusconi, F.; Fourgeaud, M.; Dubost, L.; Moumi, A. N.; Delfosse, V.; Mayer, C.; Gutmann, L.; Rice, L. B.; Arthur, M., Unexpected inhibition of peptidoglycan LD-transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. *J Biol Chem* **2007**, *282* (42), 30414-22.
9. Mainardi, J. L.; Legrand, R.; Arthur, M.; Schoot, B.; van Heijenoort, J.; Gutmann, L., Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in *Enterococcus faecium*. *J Biol Chem* **2000**, *275* (22), 16490-6.
10. Mainardi, J. L.; Morel, V.; Fourgeaud, M.; Cremniter, J.; Blanot, D.; Legrand, R.; Frehel, C.; Arthur, M.; Van Heijenoort, J.; Gutmann, L., Balance between two transpeptidation mechanisms determines the expression of beta-lactam resistance in *Enterococcus faecium*. *J Biol Chem* **2002**, *277* (39), 35801-7.
11. Hugonnet, J. E.; Mengin-Lecreulx, D.; Monton, A.; den Blaauwen, T.; Carbonnelle, E.; Veckerle, C.; Brun, Y. V.; van Nieuwenhze, M.; Bouchier, C.; Tu, K.; Rice, L. B.; Arthur, M., Factors essential

- for L,D-transpeptidase-mediated peptidoglycan cross-linking and beta-lactam resistance in *Escherichia coli*. *Elife* **2016**, 5, e19469.
12. Both, D.; Steiner, E. M.; Stadler, D.; Lindqvist, Y.; Schnell, R.; Schneider, G., Structure of LdtMt2, an L,D-transpeptidase from *Mycobacterium tuberculosis*. *Acta Crystallogr D Biol Crystallogr* **2013**, 69 (Pt 3), 432-41.
13. Correale, S.; Ruggiero, A.; Capparelli, R.; Pedone, E.; Berisio, R., Structures of free and inhibited forms of the L,D-transpeptidase Ldt_{Mt1} from *Mycobacterium tuberculosis*. *Acta Crystallogr D Biol Crystallogr* **2013**, 69 (Pt 9), 1697-706.
14. Li, W. J.; Li, D. F.; Hu, Y. L.; Zhang, X. E.; Bi, L. J.; Wang, D. C., Crystal structure of L,D-transpeptidase Ldt_{Mt2} in complex with meropenem reveals the mechanism of carbapenem against *Mycobacterium tuberculosis*. *Cell Res* **2013**, 23 (5), 728-31.
15. Biarrotte-Sorin, S.; Hugonnet, J. E.; Delfosse, V.; Mainardi, J. L.; Gutmann, L.; Arthur, M.; Mayer, C., Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. *J Mol Biol* **2006**, 359 (3), 533-538.
16. Mainardi, J. L.; Fourgeaud, M.; Hugonnet, J. E.; Dubost, L.; Brouard, J. P.; Ouazzani, J.; Rice, L. B.; Gutmann, L.; Arthur, M., A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. *J Biol Chem* **2005**, 280 (46), 38146-38152.
17. Goffin, C.; Ghuysen, J. M., Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. *Microbiol Mol Biol Rev* **2002**, 66 (4), 702-38, table of contents.
18. Lecoq, L.; Dubee, V.; Triboulet, S.; Bougault, C.; Hugonnet, J. E.; Arthur, M.; Simorre, J. P., Structure of *Enterococcus faecium* L,D-transpeptidase acylated by ertapenem provides insight into the inactivation mechanism. *ACS Chem Biol* **2013**, 8 (6), 1140-6.
19. Magnet, S.; Arbeloa, A.; Mainardi, J. L.; Hugonnet, J. E.; Fourgeaud, M.; Dubost, L.; Marie, A.; Delfosse, V.; Mayer, C.; Rice, L. B.; Arthur, M., Specificity of L,D-transpeptidases from gram-positive bacteria producing different peptidoglycan chemotypes. *J Biol Chem* **2007**, 282 (18), 13151-9.
20. Tipper, D. J.; Strominger, J. L., 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* **1965**, 54 (4), 1133-41.
21. Triboulet, S.; Arthur, M.; Mainardi, J. L.; Veckerle, C.; Dubee, V.; Nguekam-Nouri, A.; Gutmann, L.; Rice, L. B.; Hugonnet, J. E., Inactivation kinetics of a new target of beta-lactam antibiotics. *J Biol Chem* **2011**, 286 (26), 22777-84.
22. Bhattacharjee, N.; Field, M. J.; Simorre, J. P.; Arthur, M.; Bougault, C. M., Hybrid potential simulation of the acylation of *Enterococcus faecium* L,D-transpeptidase by carbapenems. *J Phys Chem B* **2016**, 120 (21), 4767-81.
23. Dubee, V.; Triboulet, S.; Mainardi, J. L.; Etheve-Quelquejeu, M.; Gutmann, L.; Marie, A.; Dubost, L.; Hugonnet, J. E.; Arthur, M., Inactivation of *Mycobacterium tuberculosis* L,d-transpeptidase LdtMt(1) by carbapenems and cephalosporins. *Antimicrob Agents Chemother* **2012**, 56 (8), 4189-95.
24. Dhar, N.; Dubée, V.; Ballell, L.; Cuinet, G.; Hugonnet, J.-L.; Signorino-Gelo, F.; Barros, D.; Arthur, M.; McKinney, J., Rapid cytolysis of *Mycobacterium tuberculosis* by faropenem, an orally bioavailable beta-lactam antibiotic. *Antimicrob Agents Chemother* **2015**, 59, 1308-1319.
25. Gupta, R.; Lavollay, M.; Mainardi, J. L.; Arthur, M.; Bishai, W. R.; Lamichhane, G., The *Mycobacterium tuberculosis* protein Ldt(Mt2) is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. *Nat Med* **2010**, 16, 466-469.
26. Diacon, A. H.; van der Merwe, L.; Barnard, M.; von Groote-Bidlingmaier, F.; Lange, C.; Garcia-Basteiro, A. L.; Sevene, E.; Ballell, L.; Barros-Aguirre, D., beta-lactams against tuberculosis-new trick for an old dog? *N Engl J Med* **2016**, 375 (4), 393-4.
27. Iannazzo, L.; Soroka, D.; Triboulet, S.; Fonvielle, M.; Compain, F.; Dubee, V.; Mainardi, J. L.; Hugonnet, J. E.; Braud, E.; Arthur, M.; Etheve-Quelquejeu, M., Routes of synthesis of carbapenems for optimizing both the inactivation of L,D-transpeptidase Ldt_{Mt1} of

- Mycobacterium tuberculosis* and the stability toward hydrolysis by beta-Lactamase BlaC. *J Med Chem* **2016**, 59 (7), 3427-38.
28. Edoo, Z.; Iannazzo, L.; Compain, F.; Li de la Sierra Gallay, I.; van Tilbeurgh, H.; Fonvielle, M.; Bouchet, F.; Le Run, E.; Mainardi, J. L.; Arthur, M.; Etheve-Quelquejeu, M.; Hugonnet, J. E., Synthesis of avibactam derivatives and activity on beta-lactamases and peptidoglycan biosynthesis enzymes of mycobacteria. *Chemistry* **2018**, 24(32), 8081-6.
 29. Cordillot, M.; Dubee, V.; Triboulet, S.; Dubost, L.; Marie, A.; Hugonnet, J. E.; Arthur, M.; Mainardi, J. L., In vitro cross-linking of *Mycobacterium tuberculosis* peptidoglycan by L,D-transpeptidases and inactivation of these enzymes by carbapenems. *Antimicrob Agents Chemother* **2013**, 57 (12), 5940-5.
 30. Edoo, Z.; Arthur, M.; Hugonnet, J. E., Reversible inactivation of a peptidoglycan transpeptidase by a beta-lactam antibiotic mediated by beta-lactam-ring recyclization in the enzyme active site. *Sci Rep* **2017**, 7 (1), 9136.
 31. Hugonnet, J. E.; Blanchard, J. S., Irreversible inhibition of the *Mycobacterium tuberculosis* beta-lactamase by clavulanate. *Biochemistry* **2007**, 46 (43), 11998-2004.
 32. Tremblay, L. W.; Hugonnet, J. E.; Blanchard, J. S., Structure of the covalent adduct formed between *Mycobacterium tuberculosis* beta-lactamase and clavulanate. *Biochemistry* **2008**, 47 (19), 5312-6.
 33. Xu, H.; Hazra, S.; Blanchard, J. S., NX104 irreversibly inhibits the beta-lactamase from *Mycobacterium tuberculosis*. *Biochemistry* **2012**, 51 (22), 4551-7.
 34. Hugonnet, J. E.; Tremblay, L. W.; Boshoff, H. I.; Barry, C. E., 3rd; Blanchard, J. S., Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science* **2009**, 323 (5918), 1215-8.
 35. Tremblay, L. W.; Fan, F.; Blanchard, J. S., Biochemical and structural characterization of *Mycobacterium tuberculosis* beta-lactamase with the carbapenems ertapenem and doripenem. *Biochemistry* **2010**, 49 (17), 3766-73.
 36. Frere, J. M.; Ghuysen, J. M.; Iwatsubo, M., Kinetics of interaction between the exocellular DD-carboxypeptidase-transpeptidase from *Streptomyces* R61 and beta-lactam antibiotics. A choice of models. *Eur J Biochem* **1975**, 57 (2), 343-51.
 37. Bourguignon-Bellefroid, C.; Wilkin, J. M.; Joris, B.; Aplin, R. T.; Houssier, C.; Prendergast, F. G.; Van Beeumen, J.; Ghuysen, J. M.; Frere, J. M., Importance of the two tryptophan residues in the *Streptomyces* R61 exocellular DD-peptidase. *Biochem J* **1992**, 282 (Pt 2), 361-7.
 38. McDonough, M. A.; Anderson, J. W.; Silvaggi, N. R.; Pratt, R. F.; Knox, J. R.; Kelly, J. A., Structures of two kinetic intermediates reveal species specificity of penicillin-binding proteins. *J Mol Biol* **2002**, 322 (1), 111-22.
 39. Silvaggi, N. R.; Josephine, H. R.; Kuzin, A. P.; Nagarajan, R.; Pratt, R. F.; Kelly, J. A., Crystal structures of complexes between the R61 DD-peptidase and peptidoglycan-mimetic beta-lactams: a non-covalent complex with a "perfect penicillin". *J Mol Biol* **2005**, 345 (3), 521-33.
 40. Kuzmic, P., DynaFit--a software package for enzymology. *Methods Enzymol* **2009**, 467, 247-80.
 41. Soroka, D.; Ourghanlian, C.; Compain, F.; Fichini, M.; Dubée, V.; Mainardi, J.; Hugonnet, J.; Arthur, M., Inhibition of β -lactamases of mycobacteria by avibactam and clavulanate. *J Antimicrob Chemother* **2017**, 72, 1081-1088.
 42. Chow, C.; Xu, H.; Blanchard, J. S., Kinetic characterization of hydrolysis of nitrocefin, cefoxitin, and meropenem by beta-lactamase from *Mycobacterium tuberculosis*. *Biochemistry* **2013**, 52 (23), 4097-104.

Figure 1. Impact of amino acid substitutions on fluorescence kinetics. (A) Inactivation reaction scheme. E, free Ldt_{fm}; EI^{An}, amine anion; EI*, acylenzyme; I, imipenem. (B) Fluorescence variations during Ldt_{fm} inactivation. The charts show the variations in the relative concentrations of the three enzyme forms (left panel) and the relative fluorescence intensity of the three enzyme forms (middle panel), which account for the observed fluorescence kinetics (right panel). (C) Fluorescence kinetics of Ldt_{fm} inactivation by imipenem. Trp residues were replaced by Phe residues in various combinations. Designation of the remaining Trp residues: a, Trp³⁵⁵; b, Trp³⁸⁵; c, Trp⁴¹⁰; d, Trp⁴¹⁵; e, Trp⁴²⁵; f, Trp⁴³⁴. Insets present the initial decreases of fluorescence over a short timescale.

Figure 2. Inactivation efficacy and relative fluorescence intensity of Ldt_{fm} and derivatives with Trp to Phe and His to Ala substitutions. (A) Kinetic parameter k_1 for formation of the amine anion. (B) Kinetic parameter k_2 for the acylation step. Error bars represent standard deviations from the fitted curves. (C) Fluorescence intensity of the three enzyme forms relative to that of the free form of the wild-type enzyme. E, Ldt_{fm} ; E^{An} , amine anion; E^* , acylenzyme. All, presence of the full complement of Trp residues (a, d, c, d, e, and f). Trp residues were replaced by Phe residues in various combinations. Designation of the remaining Trp residues: a, Trp³⁵⁵; b, Trp³⁸⁵; c, Trp⁴¹⁰; d, Trp⁴¹⁵; e, Trp⁴²⁵; f, Trp⁴³⁴.

Figure 3. Variations in fluorescence intensity resulting from acylation of L,D-transpeptidase Ldt_{fm} and β-lactamase BlaC by various β-lactams and β-lactamase inhibitors. Stopped-flow fluorescence kinetics were obtained with final concentrations of 10 μM for the enzymes and 100 μM for β-lactams and β-lactamase inhibitors (A) The negative charge developing on the amine nitrogen upon acylation of Ldt_{fm} by nitrocefin is stabilized by a mesomeric effect involving the electron withdrawing dinitrobenzene ^{30, 42}. (B) Transfer of the negative charge to a leaving group in the concerted mechanism leading to acylation of Ldt_{fm} by ceftriaxone ⁷. (C) Tautomeric forms of the acylenzyme resulting from acylation of BlaC by clavulanate and decarboxylation of the β-lactamase inhibitor ^{32, 41}. (D) BlaC-avibactam carbamoylenzyme ³³. (E and F) Acylation of BlaC and Ldt_{fm} by faropenem, respectively ²⁴. (G) Accumulation of an amine anion in the onset of the reaction of hydrolysis of meropenem by BlaC (BlaC^{An}). This is followed by protonation of the β-lactam nitrogen ³⁵.