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Inès Hugonneau-Beaufet, Jean-Philippe Barnier, Stanislas Thiriet-Rupert, Sylvie Letoffe, Jean-Luc Mainardi, et al.. Characterization of *Pseudomonas aeruginosa* L,D-transpeptidases and evaluation of their role in peptidoglycan adaptation to biofilm growth. 2023. hal-04112100v1

HAL Id: hal-04112100

<https://hal.science/hal-04112100v1>

Preprint submitted on 31 May 2023 (v1), last revised 6 Jun 2023 (v2)

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**Characterization of *Pseudomonas aeruginosa* L,D-transpeptidases
and evaluation of their role in peptidoglycan adaptation to biofilm
growth**

**Inès Hugonneau-Beaufet^a, Jean-Philippe Barnier^{a,b,c}, Stanislas Thiriet-Rupert^d, Sylvie
Létoffé^d, Jean-Luc Mainardi^{a,b,c}, Jean-Marc Ghigo^d, Christophe Beloin^{*d}, Michel Arthur^{*a}**

^a Centre de Recherche des Cordeliers, Sorbonne Université, INSERM, Université Paris Cité,
F-75006 Paris France

^b Service de Microbiologie, Hôpital Européen Georges Pompidou, AP-HP Assistance Publique-
Hôpitaux de Paris, F-75015 Paris France

^c Université Paris Cité, Faculté de Santé, UFR de Médecine, F-75015 Paris France

^d Institut Pasteur, Université Paris Cité, UMR CNRS 6047, Genetic of Biofilms laboratory, F-
75015 Paris France

^{*}Corresponding authors

Running title: *Pseudomonas aeruginosa* L,D-transpeptidases

ABSTRACT

Peptidoglycan is an essential component of the bacterial cell envelope that sustains the turgor pressure of the cytoplasm, determines cell shape, and acts as a scaffold for the anchoring of envelope polymers such as lipoproteins. The final cross-linking step of peptidoglycan polymerization is performed by classical D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family and by L,D-transpeptidases (LDTs), which are dispensable for growth in most bacterial species and whose physiological functions remain elusive. In this study, we investigated the contribution of LDTs to cell envelope synthesis in *Pseudomonas aeruginosa* grown in planktonic and biofilm conditions. We first assigned a function to each of the three *P. aeruginosa* LDTs by gene inactivation in *P. aeruginosa*, heterospecific gene expression in *Escherichia coli*, and, for one of them, direct determination of its enzymatic activity. We found that the three *P. aeruginosa* LDTs catalyze peptidoglycan cross-linking (Ldt_{Pae1}), the anchoring of lipoprotein Oprl to the peptidoglycan (Ldt_{Pae2}), and the hydrolysis of the resulting peptidoglycan-Oprl amide bond (Ldt_{Pae3}). Construction of a phylogram revealed that LDTs performing each of these three functions in various species cannot be assigned to evolutionary distinct lineages, in contrast to what has been observed with PBPs. We showed that biofilm, but not planktonic, bacteria displayed an increase proportion of peptidoglycan cross-links formed by Ldt_{Pae1} and a higher extent of Oprl anchoring to peptidoglycan, which is controlled by Ldt_{Pae2} and Ldt_{Pae3}. Consistently, deletion of each of the *ldt* gene impaired biofilm formation and potentiated the bactericidal activity of ethylenediaminetetraacetic acid (EDTA). These results indicate that LDTs contribute to the stabilization of the bacterial cell envelope and to the adaptation of peptidoglycan metabolism to growth in biofilm.

Importance. Active-site cysteine LDTs form a functionally heterologous family of enzymes that contribute to the biogenesis of the bacterial cell envelope through formation of peptidoglycan cross-links and through the dynamic anchoring of lipoproteins to peptidoglycan. Here, we report the role of three *P. aeruginosa* LDTs that had not been previously characterized. We show that these enzymes contribute to resistance to the bactericidal activity of EDTA and to the adaptation of cell envelope polymers to conditions that prevail in biofilms. These results indicate that LDTs should be considered as putative targets in the development of drug-EDTA associations for the control of biofilm-related infections.

INTRODUCTION

Peptidoglycan (PG) is a major component of the cell envelope present in almost all bacterial species. This complex heteropolymer is composed of linear glycan chains cross-linked by short stem peptides to form a three-dimensional network that surrounds the cytoplasmic membrane and provides resistance to the turgor pressure of the cytoplasm (1). The glycan chains are formed by alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked together by β -1,4 glycosidic bonds. In Gram-negative bacteria, the D-lactoyl group of MurNAc residues are linked to a conserved peptide stem containing an L-alanine at the first position (L-Ala¹), a D-glutamic acid at the second position (D-Glu²), a diaminopimelic acid at the third position (DAP³), and two D-alanine at the fourth and fifth positions (D-Ala⁴ and D-Ala⁵). In Gram-positive bacteria, DAP³ is often amidated or replaced by an L-Lysine (L-Lys³), which can be substituted by a short side chain (*e.g.* a pentaglycine in *Staphylococcus aureus*).

The peptide stems of two adjacent subunits are linked together by peptide cross-links formed by transpeptidases. Most cross-links connect D-Ala⁴ in an acyl donor stem peptide to

DAP³ in an acyl acceptor stem peptide. Synthesis of these so-called 4→3 cross-links is catalyzed by the D,D-transpeptidase activity of Penicillin-Binding Proteins (PBPs) in two steps (2). First, the catalytic serine of PBPs attacks the carbonyl of D-Ala⁴ in a donor stem pentapeptide resulting in the formation of an acyl-enzyme and release of D-Ala⁵. Second, the ester bond of the acyl-enzyme is attacked by the side-chain amine of the residue at the 3rd position of an acceptor stem peptide, resulting in the formation of a 4→3 cross-linked peptidoglycan dimer and the release of the PBP (2).

In certain bacteria, peptidoglycan cross-linking is additionally performed by a second family of enzymes, the L,D-transpeptidases (LDTs), which connect residues at the 3rd position of the donor and acceptor stems (3→3 cross-links) (Reaction A in Fig. 1) (3, 4). Similar to PBPs, LDTs catalyze peptidoglycan cross-linking by a two-step mechanism involving formation of an acyl-enzyme. However, the acyl donor used by LDTs harbors a tetrapeptide stem instead of a pentapeptide stem. The tetrapeptide-containing substrate of LDTs is formed by hydrolysis of the D-Ala⁴-D-Ala⁵ amide bond of pentapeptide stems by D,D-carboxypeptidases (5–7). LDTs and PBPs also differ by the catalytic nucleophile, Cys *versus* Ser, and the two enzyme families are structurally unrelated (8). PBPs and LDTs are inactivated through acylation of their nucleophiles. PBPs are potentially inactivated by all classes of β-lactams. In contrast, LDTs are effectively inactivated only by β-lactams belonging to the carbapenem class (9–13).

Since the first characterization of an LDT in a highly ampicillin-resistant mutant of *Enterococcus faecium* selected *in vitro* (4), numerous members of the LDT family have been identified in Gram-positive and Gram-negative bacteria as well as in mycobacteria (14–17). The number of LDTs varies between bacterial species, ranging from one in *Neisseria meningitidis* and *Helicobacter pylori* to 21 in *Bradyrhizobium japonicum* (18). A wide variety of

functions has been associated with these enzymes that can be classified in three groups as follows.

A first group of LDTs forms 3→3 peptidoglycan cross-links and comprises enzymes with various physiological functions (Reaction A in Fig. 1). Certain LDTs participate in the maturation of peptidoglycan in *Mycobacterium smegmatis* (19, 20) and in β-lactam resistance in mutants of *Escherichia coli* and *E. faecium* selected *in vitro* (4, 7). In the latter mutants, the LDTs can fully replace the PBPs resulting in a peptidoglycan exclusively containing 3→3 cross-links. The proportion of 3→3 cross-links formed by LDTs is highly variable in wild-type bacteria, ranging from 70-80% in *Clostridioides difficile* (14) and in mycobacteria (21, 22), to <10 % in most species, including in *E. coli* (23). LDTs are essential for virulence in *Mycobacterium tuberculosis* (24) but fully dispensable in *E. coli*, at least for growth in laboratory conditions. In the latter bacterium, the proportion of 3→3 cross-links increases in the stationary phase of growth but this observation has not been associated with any phenotypic property (25). In *E. coli*, 3→3 cross-link formation was reported to participate in peptidoglycan remodeling, thereby increasing the overall robustness of the bacterial cell envelope in response to defects in the outer membrane (26). In *Salmonella enterica* serovar Typhi, an L,D-transpeptidase plays an essential role in typhoid toxin secretion (27). The enzyme edits the peptidoglycan, *i.e.* enriches peptidoglycan at the cell poles in 3→3 cross-links, thereby enabling specific cleavage of these cross-links by a specialized muramidase for translocation of the toxin through the peptidoglycan layer. This subsequently enables the release of the toxin through the outer membrane.

A second group of LDTs is specialized in the covalent anchoring of proteins to peptidoglycan (Reaction B in Fig. 1). In *E. coli*, three LDTs anchor the Braun lipoprotein (Lpp) providing a link between the peptidoglycan and the outer membrane that is thought to

contribute to the stability of the envelope (28–30). In *Coxiella burnetii*, the cell envelope was proposed to be similarly stabilized by the anchoring of an outer membrane barrel protein to peptidoglycan by an LDT during the stationary phase of growth (31).

A third group of LDTs comprises enzymes acting as hydrolases (Reaction C in Fig. 1). In *E. coli*, a member of the LDT family, YafK also known as DpaA, was found to exclusively display hydrolytic activity for cleavage of the amide bond connecting Lpp to tripeptide stems (32, 33). In combination with LDTs that anchor Lpp, this enzyme may dynamically control the equilibrium between the free and peptidoglycan-linked forms of Lpp.

In addition to the three types of amide-bond forming and hydrolyzing activities depicted as reactions A, B, and C in Fig. 1, LDTs catalyze the exchange of the terminal D-Ala⁴ of tetrapeptide stems by glycine and various D-amino (*e.g.* D-Met) and D-2-hydroxy (*e.g.* D-lactate) acids (3). For the latter reactions, the acyl-enzyme is attacked by the amine or hydroxyl group of free amino or 2-hydroxy acids. This exchange reaction results in the incorporation of D-amino acids in the peptidoglycan leading to toxic effects. In *Vibrio cholerae*, non-canonical D-amino acids promote remodeling of peptidoglycan in stationary phase and participate in the control of PG abundance and strength (34).

Attack of the acyl-enzyme by a water molecule results in the hydrolysis of the DAP³-D-Ala⁴ amide bond. In *Acinetobacter baumannii*, a hydrolytic member of the LDT family acts as an L,D-carboxypeptidase trimming off D-Ala⁴ of tetrapeptide stems thereby contributing to peptidoglycan recycling (35).

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen, responsible for both acute respiratory infections, in particular ventilator acquired pneumonia, and chronic lung infections in patients with cystic fibrosis and chronic obstructive pulmonary disease. The

ability of *P. aeruginosa* to form biofilms is critical in the pathophysiology of these infections (36–38).

In this study, we show that the chromosome of *P. aeruginosa* harbors three genes encoding members of the LDT family. We report a functional characterization of each of these three enzymes and the consequences of *ldt* gene deletions on antibiotic susceptibility, envelope stability, and *in vitro* formation of biofilms by *P. aeruginosa*.

RESULTS

***P. aeruginosa* and *E. coli* share a common peptidoglycan structure.** Our first objective was to determine the structure of peptidoglycan isolated from a stationary phase culture of *P. aeruginosa* strain PA14. Peptidoglycan was extracted, digested with muramidases, and the resulting muropeptides were separated by *rp*HPLC (Fig. 2A). Mass spectrometry analysis (Fig. 2B and Supplementary Table S1) was performed on the material that was collected in each of the individually collected peaks (peaks 1 to 7 in Fig. 2). The most abundant stem peptide was a tetrapeptide (L-Ala-D-iGlu-DAP-D-Ala), both in monomers and in the acceptor stem of dimers. The pentapeptide stem (L-Ala-D-iGlu-DAP-D-Ala-D-Ala) was not detected, indicating that the terminal D-Ala of stem pentapeptides that did not participate in peptidoglycan cross-linking were effectively trimmed off by D,D-carboxypeptidases. Cleavage of DAP-D-Ala amide bonds by L,D-carboxypeptidases or by endopeptidases generated the tripeptide stem L-Ala-D-iGlu-DAP. Most of the dimers (87%) contained 4→3 cross-links made by the D,D-transpeptidase activity of PBPs. The remaining dimers (13%) contained 3→3 cross-links formed by L,D-transpeptidases. Detection of the tripeptide L-Ala-D-iGlu-DAP linked to a Lys-Arg dipeptide (Tri→KR) revealed the anchoring of the OprI lipoprotein to the peptidoglycan (Reaction B in Fig. 1). OprI is a homolog (24.8 % identity) of the Lpp Braun lipoprotein of *E. coli*

(Supplementary Fig. S1). The disaccharide moiety was composed exclusively of GlcNAc and reduced MurNAc. De-acylated sugars, as previously reported (39, 40), were not detected, indicating that the corresponding muropeptides were present in insufficient amounts to be identified in our study design. Together, these results showed that all muropeptides detected in *E. coli* (23) are present in *P. aeruginosa* PA14. Conversely, no additional structure was detected in *P. aeruginosa*. Quantitatively, the muropeptide compositions were also very similar indicating that the peptidoglycan structure is conserved in *P. aeruginosa* and *E. coli*.

Heterospecific expression of *P. aeruginosa* *ldt* genes in *E. coli* reveals the function of the corresponding LDTs. Our next objective was to identify the LDTs catalyzing the formation of 3→3 cross-linked muropeptides 4 and 6 (depicted in Fig. 2) and the anchoring of OprI to the peptidoglycan (Reactions A and B in Fig. 1, respectively). Amino acid sequence comparisons using BlastP as the software and LDTs from *E. coli* as the queries identified three proteins comprising an YkuD L,D-transpeptidase domain (Protein family domain PF03734) with a conserved [S/T]xGCh[R/N] catalytic domain, in which C is the Cys nucleophile, x any residue, and h a hydrophobic residue (Supplementary Fig. S2). The function of these enzymes, designated Ldt_{Pae1} (PA14_54810), Ldt_{Pae2} (PA14_27180), and Ldt_{Pae3} (PA14_15840), was investigated by expressing the corresponding genes in a derivative of *E. coli* BW25113 obtained by deletion of the complete set of the six *E. coli* *ldt* genes (26), hereafter designated as *E. coli* $\Delta 6ldt$. The structure of peptidoglycan from this strain and derivatives independently producing each of the three LDTs from *P. aeruginosa* was determined based on purification of muropeptides by *rp*HPLC and determination of their structure by mass spectrometry.

The peptidoglycan of *E. coli* $\Delta 6ldt$ used for heterospecific *ldt* gene expression contained two main muropeptides, a tetrapeptide monomer and a 4→3 cross-linked Tetra→Tetra dimer formed by PBPs. Expression of *P. aeruginosa* Ldt_{Pae1} led to the formation of two additional 3→3

cross-linked dimers containing a tripeptide donor stem and a tripeptide or a tetrapeptide acceptor stem (Tri→Tri and Tri→Tetra dimers; muropeptides 4 and 6 in Fig. 2B; Table 1). These results indicate that Ldt_{Pae1} is functional in *E. coli* and acts as a peptidoglycan cross-linking enzyme in this host.

Heterospecific expression of genes encoding Ldt_{Pae2} in *E. coli* Δ 6/*ldt* led to the formation of an additional disaccharide-tripeptide monomer substituted by the dipeptide Lys-Arg (KR; muropeptide 3 in Fig. 2B; Table 1). This observation indicates that Ldt_{Pae2} catalyzes the anchoring of Lpp despite the sequence divergence between the sequence of the *E. coli* (Lpp) and *P. aeruginosa* (OprI) lipoproteins (24.8% identity) (Supplementary Fig. S1).

Heterospecific production of Ldt_{Pae3} in *E. coli* Δ 6/*ldt* did not lead to any modification of the muropeptide profile. Thus, Ldt_{Pae3} did not catalyze formation of 3→3 cross-links or the anchoring of the Braun lipoprotein in *E. coli*.

Deletion of *P. aeruginosa* *ldt* genes confirms the function of Ldt_{Pae1}, Ldt_{Pae2}, and Ldt_{Pae3} inferred from heterospecific gene expression. *rp*HPLC chromatography and mass spectrometry analyses revealed that deletion of *ldt_{Pae1}* abolished the formation of 3→3 cross-linked dimers, indicating that Ldt_{Pae1} is the only peptidoglycan cross-linking L,D-transpeptidase produced by *P. aeruginosa* PA14 (Table 2). Deletion of *ldt_{Pae2}* alone abolished OprI anchoring indicating that Ldt_{Pae2} is the only enzyme responsible for the anchoring of that lipoprotein. In comparison with the parental strain, deletion of *ldt_{Pae3}* did not result in any modification of the muropeptides. Deletion of the three *ldt* genes abolished both the formation of 3→3 cross-links and the anchoring of the Lpp lipoprotein. Deletion of the *oprI* gene prevented formation of muropeptide 3 (Tri→KR; M_{cal} = 1,154.567), confirming that this muropeptide exclusively originates from anchoring of OprI to peptidoglycan. Together, these results (Table 2) indicate that formation of 3→3 cross-linked dimers and lipoprotein anchoring is mediated by Ldt_{Pae1}

and Ldt_{Pae2}, respectively, whereas Ldt_{Pae3} catalyzes neither reaction, in agreement with the analysis of heterospecific expression of *P. aeruginosa ldt* genes in *E. coli* $\Delta 6ldt$ (above, Table 1).

Purified Ldt_{Pae1} catalyzes the formation of 3→3 cross-linked dimers. In order to validate the catalytic activity of Ldt_{Pae1}, we produced a soluble fragment of Ldt_{Pae1} lacking the membrane anchor (residues 1 to 32) in *E. coli* and purified by metal affinity and size-exclusion chromatography. Consistent with our previous results, Ldt_{Pae1} was functional in the formation of 3→3 cross-linked dimers (Fig. 3) using tetrapeptide-containing peptidoglycan fragments as substrates (Muropeptide 2 in Fig. 2B). The purified protein also catalyzed the exchanges of D-Ala⁴ of these substrates by D-Met (Fig. 3). Ldt_{Pae1} formed covalent links with β -lactams representative of the cephem (ceftriaxone) and carbapenem (meropenem) classes but not with the penam ampicillin. This β -lactam specificity was previously observed for LDTs from various bacteria (9–13, 41). We could not similarly investigate the catalytic activity of Ldt_{Pae2} and Ldt_{Pae3} since expression of fragments of the *ldt*_{Pae2} and *ldt*_{Pae3} genes in the conditions reported for *ldt*_{Pae1} did not afford soluble proteins.

Ldt_{Pae1} is unable to bypass β -lactam-inactivated PBPs. We have previously shown that the D,D-transpeptidase activity of all PBPs can be replaced by the L,D-transpeptidase activity of one of the six LDTs of *E. coli*, namely YcbB (7). This results in broad-spectrum β -lactam resistance because YcbB is not effectively inactivated by β -lactams belonging to the penicillin and cephalosporin classes. In the presence of these drugs, all (>95%) of the peptidoglycan cross-links are of the 3→3 type indicating that the PBPs do not contribute to peptidoglycan polymerization under such conditions. The bypass resistance mechanism also requires overproduction of the (p)ppGpp alarmone to prevent the bacterial killing triggered by inactivation of the transpeptidase domains of the PBPs (7, 42). This bactericidal activity of β -lactams is

233 thought to result from the uncoupling of the transglycosylation and transpeptidation reactions
234 thereby leading to the accumulation of uncross-linked glycan chains in the periplasm and the
235 activation of a futile cycle of glycan chain polymerization and hydrolysis (42). Experimentally,
236 overproduction of (p)ppGpp is achieved in an *E. coli* BW25113 derivative by the introduction
237 of plasmid pKT8(*relA'*) encoding an unregulated (p)ppGpp synthase (*RelA'*) and by the deletion
238 of the chromosomally-located *relA* gene encoding the wild-type (p)ppGpp synthase (7).
239 Although L,D-transpeptidase YnhG from *E. coli* also catalyzes formation of 3→3 cross-links,
240 this enzyme is unable to confer β-lactam resistance in the host overproducing (p)ppGpp (7).
241 We therefore investigate whether Ldt_{Pae1}, which forms 3→3 cross-links, was able to bypass
242 PBPs and confer β-lactam resistance in *E. coli*. To address this question, we introduced plasmid
243 pIHB1, which carries a copy of the *ldt_{Pae1}* gene under the control of an IPTG-inducible
244 promoter, in the derivative of *E. coli* BW25113 that overproduces the (p)ppGpp alarmone.
245 Plasmids coding for inducible production of YcbB and YnhG were used as positive and negative
246 controls, respectively. Production of *P. aeruginosa* Ldt_{Pae1} did not mediate β-lactam resistance
247 in this host as previously found for *E. coli* YnhG (Table 3). Thus, YnhG and Ldt_{Pae1} were unable
248 to cross-link a functional peptidoglycan under conditions in which the D,D-transpeptidase
249 activity of PBPs was inactivated by β-lactams, even though both proteins are functional LDTs
250 for synthesis of 3→3 peptidoglycan cross-links *in vitro* and in *E. coli*. Similar to PBPs involved
251 in peptidoglycan cross-linking, YcbB is a high-molecular weight protein composed of 615
252 residues that contains several domains not present in other LDTs, including LDT_{Pae1} and YnhG
253 (Supplementary Fig. 4). These domains are likely to be essential for the bypass of PBPs by
254 enabling YcbB to recruit accessory proteins required to carry out peptidoglycan cross-linking
255 during the entire cell cycle. These accessory proteins may include the bifunctional
256 transpeptidase-glycosyltransferase class A PBP1b, which displays affinity for YcbB (26), and

scaffolding proteins controlling the sites of peptidoglycan expansion during the cell cycle (7, 42).

The activity of β -lactams is not affected by the deletion of *ldt* genes. The activity of 31 antibiotics, including 20 β -lactams, was tested by the disk diffusion assay against *P. aeruginosa* PA14 and isogenic derivatives obtained by deletion of L,D-transpeptidase genes (Supplementary Table S3). Deletion of *ldt*_{Pae1}, *ldt*_{Pae2} plus *ldt*_{Pae3}, or of the three L,D-transpeptidase genes did not affect the susceptibility of *P. aeruginosa* to any of the tested antibiotics. Thus, the L,D-transpeptidases did not contribute to growth of PA14 in the presence of sub-inhibitory concentrations of β -lactams or to the efficacy of the permeability barrier mediated by the outer membrane.

Deletion of *ldt*_{Pae2} destabilizes the *P. aeruginosa* envelope. Ethylenediaminetetraacetic acid (EDTA) is often used to probe the stability of the outer membrane of *E. coli* since this compound prevents the stabilization of the outer leaflet of the outer membrane by Mg²⁺ cations (30, 42). Incubation of *P. aeruginosa* PA14 derivatives lacking *oprI* or *ldt*_{Pae2} resulted in similar kinetics of bacterial killing by EDTA with *ca.* 6 log₁₀ decreases of the initial inoculum (Fig. 4). Deletion of *ldt*_{Pae1} or of *ldt*_{Pae3} had no impact on killing in comparison to wild-type PA14 with < 2 log₁₀ decreases of the initial inoculum. Thus, loss of OprI or of the anchoring of this lipoprotein to the peptidoglycan destabilizes the cell envelope leading to cell death in the presence of EDTA.

Deletion of *ldt* genes impairs biofilm formation but does not affect motility. Screening of a library of mutants obtained by random insertions of a transposon previously revealed that a member of the LDT family (YafK) is required for biofilm formation by an enteroaggregative strain of *Escherichia coli* (43). This prompted us to determine the impact of *ldt* deletions on biofilm formation by *P. aeruginosa*.

Our first objective was to evaluate the contribution of LDTs to peptidoglycan synthesis in biofilm (Supplementary Table S2 and Fig. 5). The proportion of 3→3 cross-linked dimers among all dimers was 11.3% in peptidoglycan extracted from biofilms, whereas 3→3 cross-links were not detected for exponential growth of PA14 in planktonic form (Fig. 5B). The relative proportion of dimers among all muropeptides was higher in biofilm than in planktonic cultures (44.2% *versus* 33.9%) (Fig. 5A). These results indicate that growth in biofilm favors peptidoglycan cross-linking by Ldt_{Pae1} and that this growth condition was associated with a highly cross-linked peptidoglycan. Growth in biofilm also favored the anchoring of Oprl to peptidoglycan indicating that Ldt_{Pae2} was active in this growth condition (Fig. 5C).

Our second objective was to determine whether the activity of the LDTs modulate biofilm formation. The deletion of *ldt*_{Pae1}, *ldt*_{Pae2}, *ldt*_{Pae3}, or *oprl* resulted in statistically significant but limited decreases in the capacity of *P. aeruginosa* PA14 to form biofilm in the 96-well plate assay (Fig. 5D). The combine deletions of all three L,D-transpeptidases did not further decrease biofilm formation. Additionally, we also demonstrated that deletions of *ldt* genes or of *oprl* had no impact on the swimming motility of PA14, a biofilm-related function, (Supplementary Table S4) indicating that the cell envelope of the mutants was fully compatible with the assembly of a functional flagellum.

Comparison of LDTs catalyzing various reactions. A peptidoglycan cross-linking activity was assigned to Ldt_{Pae1}, a lipoprotein anchoring activity to Ldt_{Pae2}, and neither activity to Ldt_{Pae3} (above, Tables 1 and 2). The resulting extension in the number of functionally characterized LDTs prompted us to explore the phylogenetic relationships between members of this protein family (Supplementary Table S5 and Supplementary Fig. S2 and S3). Closely related species were found to produce the same number of closely related LDTs, which share sequence identity over the entire sequences [*e.g.* *E. coli* and *Salmonella enterica* (6 paralogues), *E.*

305 *faecalis* and *E. faecium* (1 paralogue), *Mycobacterium tuberculosis* and *Mycobacterium*
306 *abscessus* (6 paralogues)]. More distantly related species, such as *E. coli* and *P. aeruginosa* or
307 *E. faecium* and *S. aureus* (which does not harbor any LDT) produce various numbers of LDTs
308 (0 to 6) and the domain composition is not conserved (Supplementary Fig. S4).

309 We then evaluated whether LDTs catalyzing similar reactions could be identified based
310 on the presence of specific sequence signatures. Winkle *et al.* identified a polymorphism in
311 the conserved catalytic motif of *E. coli* LDTs (SxGChR *versus* SxGChA) and proposed that the
312 latter motif was specific of enzymes hydrolyzing the amide bond connecting tripeptide stems
313 to lipoproteins [YafK also referred to DpaA in *E. coli*, (32, 33). Ldt_{Pae3} might be involved in the
314 releasing of anchored OprI in *P. aeruginosa* since this L,D-transpeptidase contains an SxGChA
315 motif and is closely related to YafK (Supplementary Table S5 and Supplementary Fig. S2 and
316 S3). Ldt_{Pae2} (OprI anchoring) belonged to a lineage that included both *E. coli* Lpp anchoring
317 (ErfK, Ybis, and YcfS) and peptidoglycan cross-linking (YnhG) enzymes. For Ldt_{Pae1} (formation
318 of 3→3 cross-links), the highest levels of identity were observed with YciB from *B. subtilis*
319 (35.6%; unknown function), Ldt_{Mab3} from *Mycobacterium abscessus* (32.9%; unknown
320 function), and YcfS of *Campylobacter jejuni* and *E. coli* (31.2% and 30.1 %, unknown and Lpp
321 anchoring respectively). Together, these results imply that LDTs with lipoprotein anchoring
322 and 3→3 cross-linking activities cannot be assigned to two evolutionary divergent lineages
323 because they are scattered in the various branches of the phylogram depicted in
324 Supplementary Fig S3.

325 **Ldt_{Pae1} comprises two YkuD domains.** Sequence comparison indicated that Ldt_{Pae1}, which
326 catalyzes the formation of 3→3 peptidoglycan cross-links, comprises an N-terminal *bona fide*
327 LDT catalytic domain with the conserved catalytic motif (SHGCIR) followed by a related domain
328 that lacks the catalytic Cys residue (QLGKIR) (these domains are designated Ldt_{Pae1} and Ldt_{Pae11}

respectively in Supplementary Table S5 and Supplementary Fig. S2 and S3, and YkuD1 and YkuD2 in Supplementary Fig. S4). These two domains are clustered in the same lineage suggesting a duplication. This domain architecture has not been detected in any other LDT sequence. The role of the catalytically deficient domain Ldt_{Pae11} remains to be determined. It is tempting to speculate that it could be involved in peptidoglycan binding, a function mediated by unrelated domains in other LDTs (44, 45).

DISCUSSION

Active-site cysteine L,D-transpeptidases comprising a conserved YkuD domain catalyze various reactions (Fig. 1 and Introduction section) (46). Here we show that *P. aeruginosa* may produce representatives of each of the three catalytic functions associated with the conserved YkuD catalytic domain, namely, the formation of 3→3 peptidoglycan cross-links (Ldt_{Pae1}), the anchoring of lipoprotein OprI to PG (Ldt_{Pae2}), and the hydrolysis of the resulting tripeptide→OprI amide bond (Ldt_{Pae3}). The activity of these enzymes was investigated by *in vitro* assays for purified Ldt_{Pae1} (Fig. 3), deletion of *ldt* genes from the chromosome of *P. aeruginosa* PA14 (Table 2), and heterologous expression of *ldt* genes in *E. coli* (Table 1). Functionality of Ldt_{Pae1} and Ldt_{Pae2} in the heterologous host might be accounted for by the conserved structures of both the peptidoglycan subunit of *P. aeruginosa* and *E. coli* (Fig. 2) and the Arg-Lys C-terminus of lipoproteins OprI and Lpp (Supplementary Fig. S1). The function of Ldt_{Pae3} was tentatively assigned to the release of OprI based both on the absence of detectable amide bond forming activity (formation of 3→3 and tripeptide→ OprI bonds) and on the close similarity between Ldt_{Pae3} and *E. coli* hydrolase YafK.

Phylogenetic analysis revealed that the number of LDTs and their domain composition are not conserved except in closely related bacteria belonging to the same genus

(Supplementary Table S5 and Supplementary Fig. S2, S3, and S4). LDTs with lipoprotein anchoring and 3→3 cross-linking activities cannot be assigned to two distinct evolutionary lineages. Thus, although *ldt* genes appear to belong to bacterial core genomes, they are not stably inherited in distantly related lineages. In contrast, comparison of high molecular-weight PBPs involved in peptidoglycan cross-linking clearly identifies orthologues in distantly related lineages (47).

The phenotypic impact of deletions of *ldt* genes and of *oprI* has been explored using various assays. The deletion of these genes did not result in hyper-susceptibility to antibiotics or impaired motility (Supplementary Tables S3 and S4, respectively). These observations indicate that the permeability barrier of the outer membrane and the functionality of the flagellum were preserved in the mutants. Loss of OprI or of the anchoring of this lipoprotein to PG led to a destabilization of the cell envelope, as revealed by the bactericidal effect of EDTA (Fig. 4). In contrast, formation of 3→3 cross-links by Ldt_{Pae1} was dispensable for survival in the presence of EDTA. Similar results were previously obtained for mutants of *E. coli* deficient in the production of Lpp or in the anchoring of this lipoprotein to peptidoglycan (30).

Formation of 3→3 cross-links remained undetected for exponential growth of *P. aeruginosa* PA14 in planktonic form (Fig. 5B). The proportion of 3→3 cross-links was significantly higher (11.3%) for growth in biofilm ($P < 0.01$). Deletion of *ldt*_{Pae1} had a modest but significant ($P < 0.05$) impact on the ability of *P. aeruginosa* PA14 to form biofilms (Fig. 5D). These results indicate that 3→3 cross-linking of glycan strands participates in the adaptation of peptidoglycan metabolism to conditions that prevail in biofilm. Interestingly, muropeptides containing 3→3 cross-links were found to be more abundant in *P. aeruginosa* epidemic strains than in reference strains PAO1 and PA14 (40). In addition, deletion of *ldt*_{Pae2} or *oprI* impaired biofilm formation and sensitized mutants to EDTA. Thus, L,D-transpeptidases might be

actionable targets to fight against bacteria in biofilm. Considering the impact of *ldt* gene deletions on the bactericidal activity of EDTA (Fig. 4), it would be relevant to determine whether inhibition of LDTs of Gram-negative bacteria could act in synergy with drug-EDTA associations for the eradication of catheter-associated biofilms based on antibiotic lock therapy or in treatment of other biofilm-related infections (48–50).

METHODS

Bacterial strains and plasmids. The characteristics and origin of plasmids and bacterial strains are listed in Supplementary Tables S6 and S7, respectively.

Cultures for peptidoglycan extraction. For the analyses of the muropeptide composition of the peptidoglycan from *P. aeruginosa* PA14 and derivatives obtained by deletion of *ldt* genes and *oprI*, bacteria were grown overnight to the stationary phase in 200 ml Lysogeny (Miller) broth (LB) at 37°C. For quantitative analyses of the peptidoglycan of *P. aeruginosa* PA14 in the exponential phase of growth, bacteria were grown in 1 L of LB broth and collected at an optical density at 600 nm (OD₆₀₀) of 0.8. For quantitative analyses of the peptidoglycan of *P. aeruginosa* PA14 grown in biofilm, continuous-flow biofilm microfermentors containing a removable glass spatula were used as described previously (51). Biofilm microfermentors were inoculated by placing the spatula in a culture solution adjusted to an OD₆₀₀ of 1.0 (*ca.* 5.0 × 10⁸ bacteria/mL) for 5 min. The spatula was placed into the microfermentor and biofilm culture was performed at 37° C in Miller LB broth. Flow rate was adjusted so that total time for renewal of microfermentor medium was shorter than bacterial generation time, thus minimizing planktonic growth by constant dilution of non-biofilm bacteria. Biofilms were allowed to grow on the glass spatula for 72 h after which the microfermentor was vortexed for 1 minute to resuspend the bacterial population. The resulting bacterial suspension (50 mL)

401 was centrifuged 15 min at 7,000 x *g* (4 °C) and peptidoglycan was extracted by the hot SDS
402 procedure. Peptidoglycan analyses were performed for a minimum of three biological repeats.

403 **Analysis of peptidoglycan structure.** Sacculi were extracted by the hot SDS procedure and
404 treated with pronase and trypsin (52, 7). Muropeptides were solubilized by digestion with
405 muramidases, reduced with NaBH₄, and purified by *rp*HPLC. The mass of muropeptides was
406 determined on a Bruker Daltonics maXis high resolution mass spectrometer (Bremen
407 Germany) operating in the positive mode, as previously described (53, 54).

408 **Heterospecific expression of *P. aeruginosa* *ldt* genes in *E. coli* BW25113 $\Delta 6ldt$.** Genes *ldt*_{Pae1},
409 *ldt*_{Pae2}, and *ldt*_{Pae3} were cloned into the vector pHV6 under the control of the *trc* promoter by
410 Gibson assembly. Genes encoding LDTs were induced at OD₆₀₀ = 0.2 with 100 μ M of isopropyl
411 β -D-1-thiogalactopyranoside in LB broth. The incubation was continued for 18 h at 37 °C.

412 **Purification of Ldt_{Pae1}.** A fragment of the Ldt_{Pae1} encoding residues 33 to 347 of the L,D-
413 transpeptidase was cloned into the vector pET-TEV generating a translational fusion with a C-
414 terminal 6 x His tag. The protein was produced in *E. coli* BL21 and purified from a clarified
415 lysate by metal affinity and size exclusion chromatography as previously performed for L,D-
416 transpeptidase YcbB from *E. coli* (7). Protein concentration was determined by the BioRad
417 assay using bovine serum albumin as the standard.

418 **Peptidoglycan transpeptidase activity of purified Ldt_{Pae1}.** Reduced disaccharide-tetrapeptide
419 and lactoyl-tetrapeptide were extracted from the peptidoglycan of *E. coli* BW25113 $\Delta 6ldt$ as
420 previously described (55). Purified Ldt_{Pae1} (5 μ M) was incubated with peptidoglycan fragments
421 (50 μ M) in 25 mM Tris-HCl (pH 8.0) at 20 °C. LC/MS was performed with a nucleoshell RP 18
422 column (5 μ M; 50 x 2 mm) coupled to a low-resolution LCQ Advantage mass spectrometer
423 (ThermoElectron). The reactions were also carried out in the presence of additional D-Met (1
424 mM).

425 **Mass spectrometry analyses of Ldt_{Pae1} acylation by β -lactams.** The formation of drug-enzyme
426 adducts was tested by incubating Ldt_{Pae1} (10 μ M) with β -lactams (100 μ M) at 20 °C in 5 mM
427 Tris-HCl (pH 8.0) (9). Five microliters of acetonitrile and 1 μ l of 1% formic acid were added,
428 and the reaction mixture was directly injected into the mass spectrometer Spectra were
429 acquired in the positive mode on a Bruker Daltonics maXis high resolution mass spectrometer
430 (Bremen Germany) operating in the positive mode.

431 **Deletion of genes *ldt_{Pae1}*, *ldt_{Pae2}*, *ldt_{Pae3}*, and *oprI* of *P. aeruginosa* PA14.** The two-step allelic
432 exchange procedure was used for deleting *ldt* genes and *oprI* (56). Briefly, sequences flanking
433 the genes were cloned into the vector pEX18. The plasmids were transferred by conjugation
434 from *E. coli* S17 λ pir to *P. aeruginosa* PA14. Transconjugants were selected on LB agar plates
435 containing 15 μ g/mL triclosan and 75 μ g/ml tetracycline. Colonies were subcultured in salt
436 free LB containing 5% sucrose. The genome of the mutants was sequenced by the Illumina
437 approach (Novogene, Cambridge UK).

438 **Antimicrobial susceptibility testing.** Antibigrams were performed in triplicate by the disk
439 diffusion assay in cation-adjusted Muller Hinton agar plates according to the
440 recommendations of the European Committee on Antimicrobial Susceptibility Testing
441 (EUCAST) guidelines (57).

442 **Bactericidal activity of ethylenediaminetetraacetic acid (EDTA).** Bacteria were grown
443 overnight to the stationary phase in LB (Miller) broth at 37° C, washed twice in 50 mM Tris-
444 HCl buffer (pH 8.0), and resuspended in the same buffer to a final OD₆₀₀ of 0.9. EDTA was
445 added (final concentration of 500 μ M), bacteria were incubated in 2-ml Eppendorf tubes at
446 37° C, and samples were withdrawn at 90 min, 180 min, and 270 min for viable cell counts on
447 agar plates. Data were obtained in three biological repeats.

448 **Plate-based assay for swimming motility.** An isolated colony from an overnight agar plate was
449 inoculate onto a soft agar plate containing tryptone broth (10 g/L), NaCl (5 g/L), and agar (0.3%
450 W/V) (58). Plates were incubated 18 h at 30° C and the swimming diameter was recorded.
451 Data were obtained in three biological repeats.

452 **Quantification of biofilm.** Formation of biofilm was determined with crystal violet staining in
453 96-well microtiter plates, as previously described (59). A microtiter plate was inoculated with
454 100 µl of overnight cultures at a dilution of 1:100 and incubated at 37 °C for 24 h. The medium
455 was removed and the wells were washed twice with water. 125 µl of a crystal violet solution
456 (RAL Diagnostics) was added to each well and the incubation was continued for 15 min. After
457 washing three times with water, 125 µl of a mixture ethanol and acetone (80/20; V/V) was
458 added and incubation was continued for 10 min. Absorbance was measured at 570 nm with a
459 plate reader (Tecan infinite 200Pro). Data were obtained in 24 biological repeats.

460 **Statistical analysis.** The data were analyzed using GraphPad Prism version 9 (La Jolla, CA, USA).
461 An unpaired t-test was performed to analyze the relative abundance of dimers and 3→3 cross-
462 links in the peptidoglycan of *P. aeruginosa* PA14. A Brown-Forsythe and Welch ANOVA test
463 was performed to analyze the impact of *ldt* gene deletions on bacterial killing by EDTA and the
464 quantification of biofilm formation. A $P < 0.05$ was considered statistically significant in both
465 types of tests.

466 **Sequence comparison and phylogeny.** Forty YkuD-related domains were retrieved from the
467 genome of 12 bacterial species including *E. coli* (*E. coli*_YcfS, *E. coli*_YcbB, *E. coli*_YnhG, *E.*
468 *coli*_Ybis, *E. coli*_ErfK, and *E. coli*_YafK), *P. aeruginosa* (Ldt_{Pae1} and its second YkuD domain
469 named Ldt_{Pae11}, Ldt_{Pae2}, and Ldt_{Pae3}), *Salmonella enterica* serovar Typhimurium (Sty_YcfS,
470 Sty_YcbB, Sty_YnhG, Sty_Ybis, Sty_ErfK, and Sty_YafK), *Campylobacter jejuni* (Cje_YcfS and
471 Cje_Ybis), *Neisseria meningitidis* (Ldt_{Nme}), *Bacillus subtilis* (Bs_YkuD, Bs_YqjB, and Bs_YciB),

472 *Clostridioides difficile* (Ldt_{Cd1}, Ldt_{Cd2}, and Ldt_{Cd3}), *Mycobacterium tuberculosis* (Ldt_{Mt1-6}),
473 *Mycobacterium abscessus* (Ldt_{Mab1-6}), *Enterococcus faecium* (Ldt_{fm}), *Enterococcus faecalis*
474 (Ldt_{fs}), and *Coxiella burnettii* (Ldt_{Cbu2}). The sequences of the domains were aligned using
475 Clustal Omega (Supplementary Fig. S2) providing the identity matrix reported in
476 Supplementary Table S5. The phylogram reported in Supplementary Fig. S3 was obtained on
477 iTOL (60).

478

479 **ACKNOWLEDGEMENTS**

480 High-resolution mass spectra were obtained at the Plateforme de Spectrométrie de Masse
481 Bio-organique of the Muséum d'Histoire Naturel. This work was supported by the the French
482 National Research Agency (ANR), (grant n°ANR-19-CE15-0006-01 "PeptidoAdapt", Programme
483 AAPG 2019 to MA and JM G). This work was also supported by the French government's
484 Investissement d'Avenir Program, Laboratoire d'Excellence "Integrative Biology of Emerging
485 Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID). S.T.-R was supported by the French
486 National Research Agency (ANR), project EvoTolAB (ANR-18-CE13-0010). We thank A Filloux
487 for the generous gift of *P. aeruginosa* mutants obtained by transposon insertion (full library
488 available on <http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi>).

489

FIGURE LEGENDS

Figure 1. Two-step reactions catalyzed by LDTs. In the first step, the catalytic cysteine attacks the carbonyl of D-Ala⁴ at the extremity of an acyl donor tetrapeptide stem. This results in the formation of an acyl-enzyme and the release of D-Ala⁴. In the second step, the carbonyl of the thioester bond in the acyl-enzyme is attacked by a side-chain amine harbored either by DAP³ in an acceptor stem peptide (reaction A, formation of 3→3 cross-links) or by the C-terminal L-Lys of Lpp (reaction B, Lpp anchoring). Other members of the LDT family hydrolyze the tripeptide→Lpp amide bond (reaction C, release of Lpp). Abbreviations: DAP, diaminopimelic acid; D-iGlu, D-isoglutamic acid; Lpp, major outer membrane Braun lipoprotein; FA, fatty acid.

Figure 2. Muropeptide composition of the peptidoglycan of *P. aeruginosa* strain PA14. (A) *rp*HPLC profile of muropeptides. **(B)** Structure of muropeptides. M_{obs}, observed monoisotopic mass; M_{cal}, calculated monoisotopic mass; grey hexagon, GlcNAc; blue hexagon, MurNAc; black disk, L-Ala; grey disk, D-iGlu; white disk, DAP; green disk, D-Ala; K and R circled in red, amino acid residues corresponding to the Arg-Lys C-terminal extremity of OprI.

Figure 3. Functional characterization of purified Ldt_{Pae1}. **(A)** Schematic representation of the reactions catalyzed by Ldt_{Pae1}. This enzyme forms an acylenzyme with a tetrapeptide donor stem that subsequently reacts with a tetrapeptide acyl acceptor or D-Met resulting in the formation of a 3→3 cross-linked dimer or of a tetrapeptide ending in D-Met, respectively. Ldt_{Pae1} also uses β-lactams as suicide substrates to form acylenzymes. **(B)** Identification of the reaction products by low-resolution mass spectrometry. The indicated masses are monoisotopic masses for peptidoglycan fragments and average masses for acyl-enzymes.

Tetra, reduced disaccharide-tetrapeptide, Lactoyl-Tetra, tetrapeptide linked to the D-lactoyl moiety of MurNAc following cleavage of the ether bond internal to MurNAc. D-Met, D-methionine. None, no reaction product obtained by incubation of purified Ldt_{Pae1} with ampicillin. The calculated mass of the native protein is 36,303 Da, corresponding to residues 32 to 347 fused to the MGSSHHHHHSSG His-Tag. The N-terminal methionine was not present in purified Ldt_{Pae1}.

Figure 4. Killing of *P. aeruginosa* by EDTA. Bacteria from overnight cultures were resuspended in Tris-HCl pH 8.0 and incubated with 500 μ M EDTA (open blue symbols and solid lines) or without EDTA (closed black symbols and dotted lines). Bacteria were enumerated at 0, 90, 180, and 270 min. The numbers of CFUs were normalized to the starting inoculum. Data are the mean \pm standard error of the mean from three independent biological repeats. * and **, $P < 0.05$ and $P < 0.01$; Brown-Forsythe and Welch ANOVA (wild-type with EDTA *versus* mutants with EDTA at 270 min).

Figure 5. Peptidoglycan structure of *P. aeruginosa* grown in biofilm and impact of *ldt* gene deletion on biofilm formation. (A) Proportion of dimers with 3 \rightarrow 3 cross-links among all dimers (cumulative area of peaks 4+6 / cumulative area of peaks 4+5+6+7 in *rp*HPLC chromatograms; λ =205 nm). **(B)** Proportion of dimers among all muropeptides except peak 3 (peaks 4+5+6+7 / peaks 1+2+4+5+6+7). **(C)** Proportion of muropeptides containing the Lys-Arg fragment of O_prI among all muropeptides (peak 3 / peak 1+2+3+4+5+6+7). Closed circles indicate the values obtained for biological repeats (n=3). The horizontal line indicates the mean % value. * and **, $P < 0.05$ and $P < 0.01$; unpaired t-test. BIOF, PA14 grown for 96 h in biofilm; EXPO, exponentially growing PA14 in planktonic form (OD₆₀₀ = 0.8). **(D)** Crystal violet

538 staining of biofilms formed by wild-type *P. aeruginosa* PA14 and derivatives obtained by
539 deletion of *ldt* genes and *oprI*. Circles indicate values obtained for biological repeats (n=24). *,
540 **, and ***, *P* smaller than 0.05, 0.01, and 0.001, respectively; Brown-Forsythe and Welch
541 ANOVA test.

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543

544

Table 1. Muropeptide composition of peptidoglycan extracted from derivatives of *E. coli* Δ 6*ldt* expressing *P. aeruginosa* L,D-transpeptidase genes

Muropeptide (cross-link)	Calculated mass ^a	Observed mass ^a			
		None	<i>ldt</i> _{Pae1}	<i>ldt</i> _{Pae2}	<i>ldt</i> _{Pae3}
Monomers					
Tri	870.371	870.372	870.371	870.370	870.371
Tetra	941.408	941.408	941.409	941.408	941.409
Tri→KR	1,154.567	ND	ND	1,154.567	ND
Dimers					
Tri→Tri (3→3)	1,722.731	ND	1,722.730	ND	ND
Tetra→Tri (4→3)	1,793.768	ND	1,793.765	1,793.765	ND
Tri→Tetra (3→3)	1,793.768	ND	1,793.765	ND	ND
Tetra→Tetra (4→3)	1,864.805	1,864.805	1,864.800	1,864.803	1,864.800

^a Monoisotopic mass.

ND, not detected.

Data are representatives of three biological repeats.

545

546

Table 2. Muropeptide composition of the peptidoglycan of derivatives of strain PA14 harboring various deletions

		Observed mass ^a of muropeptides for indicated deletions					
Muropeptide (cross-link)	Calculated mass ^a	<div><div>Δldt_{Pae1}</div><div>Δldt_{Pae2}</div><div>Δldt_{Pae3}</div><div>$\Delta oprI$</div></div>					
		None	Δldt_{Pae1}	Δldt_{Pae2}	Δldt_{Pae3}	Δldt_{Pae3}	$\Delta oprI$
Monomers							
Tri	870.371	870.370	870.369	870.371	870.370	870.374	870.364
Tetra	941.408	941.407	941.406	941.406	941.408	941.412	941.405
Tri→KR	1,154.567	1,154.566	1,154.565	ND	1,154.565	ND	ND
Dimers							
Tri-Tri (3→3)	1,722.731	1,722.731	ND	ND	ND	ND	ND
Tetra-Tri (4→3)	1,793.768	1,793.766	1,793.764	ND	1,793.767	ND	1,793.761
Tri-Tetra (3→3)	1,793.768	1,793.764	ND	1,793.764	1,793.766	ND	1,793.763
Tetra-Tetra (4→3)	1,864.805	1,864.802	1,864.801	1,864.799	1,864.804	1,864.818	1,864.800

^a Monoisotopic mass.

ND, not detected.

Data are representatives of three biological repeats except for strain $\Delta oprI$ (two repeats).

547

548

Table 3. Expression of L,D-transpeptidase-mediated resistance in (p)ppGpp-producing *E. coli* ^a

Plasmid	Inhibition zone (mm) around disks containing β -lactams	
	Ceftriaxone (30 μ g)	Ampicillin (10 μ g)
pHV6 (vector)	37	23
pHV6(<i>ycbB</i>)	12	≤ 6
pHV6(<i>ynhG</i>)	38	21
pHV6(<i>Idt</i> _{Pae1})	39	20

^a The *E. coli* host harbored a chromosomal deletion of *relA* and plasmid pKT8 encoding RelA'.

Data are the medians from three biological repeats.

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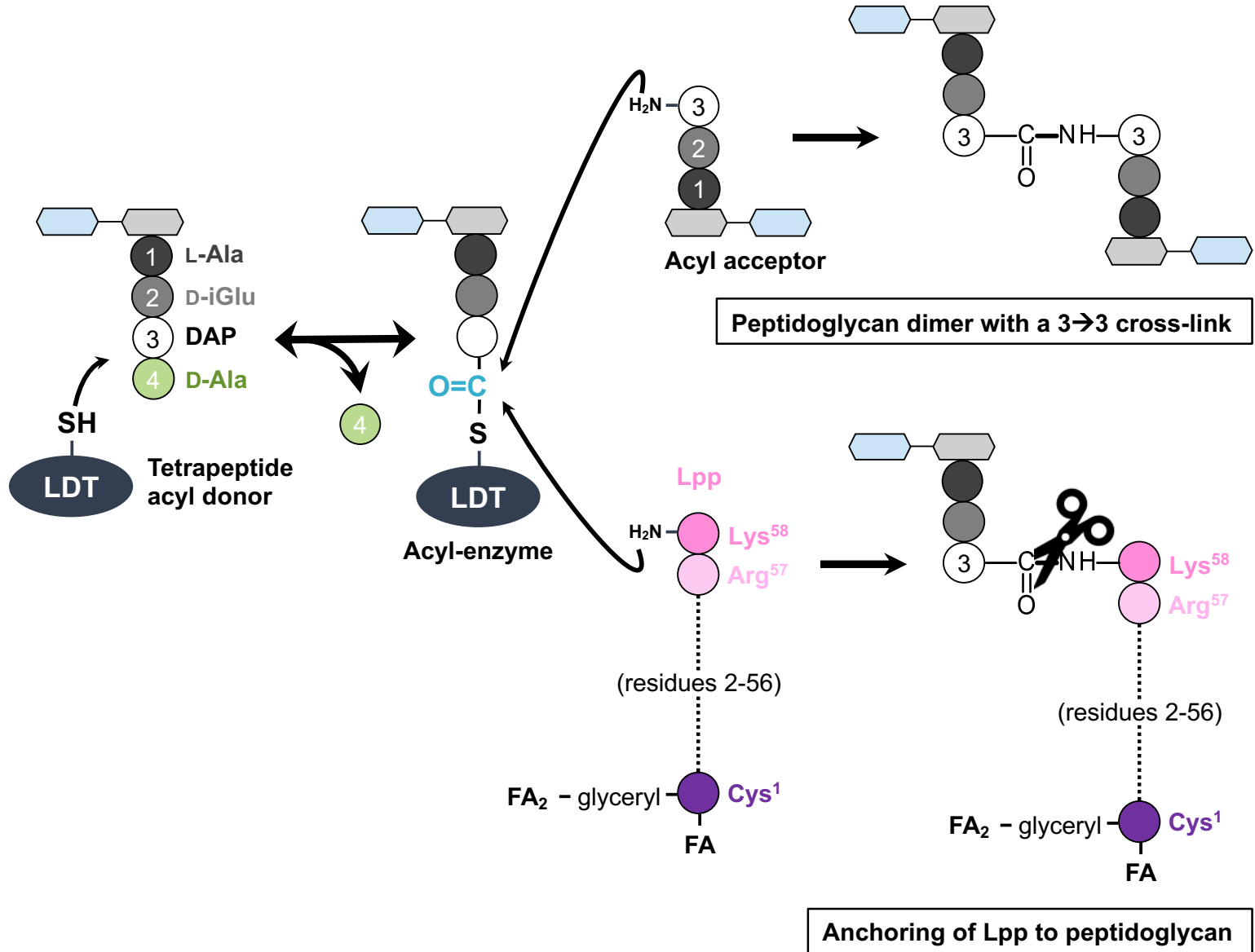
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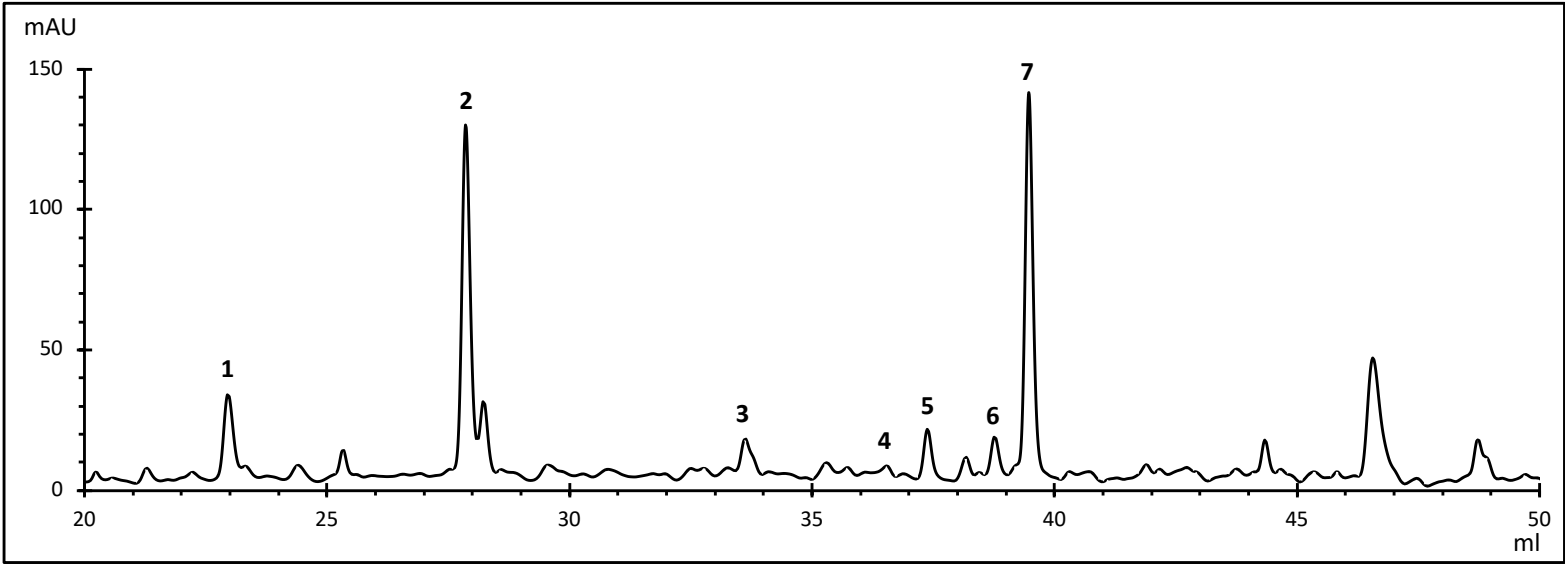
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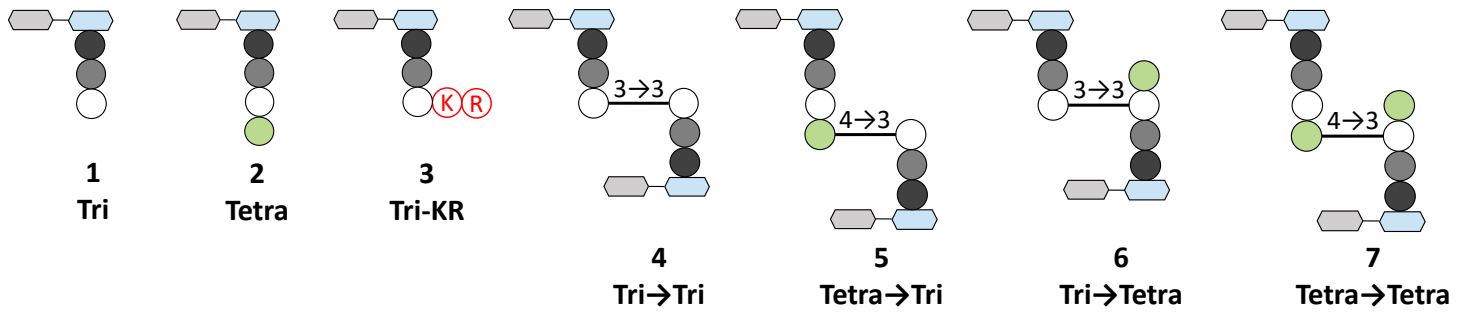
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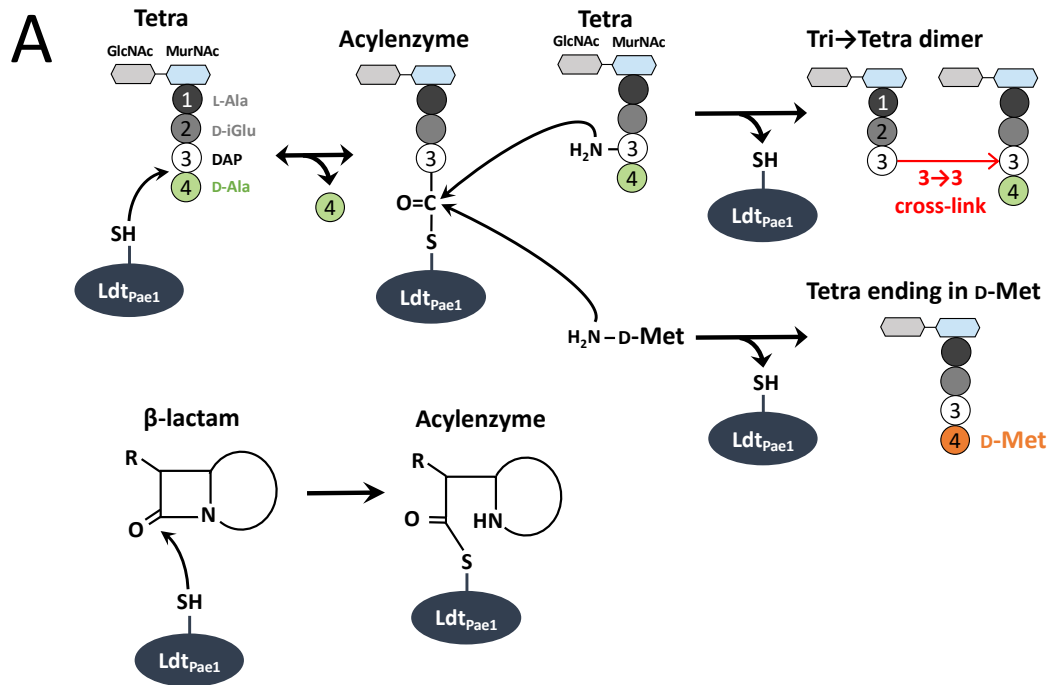
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A**B**

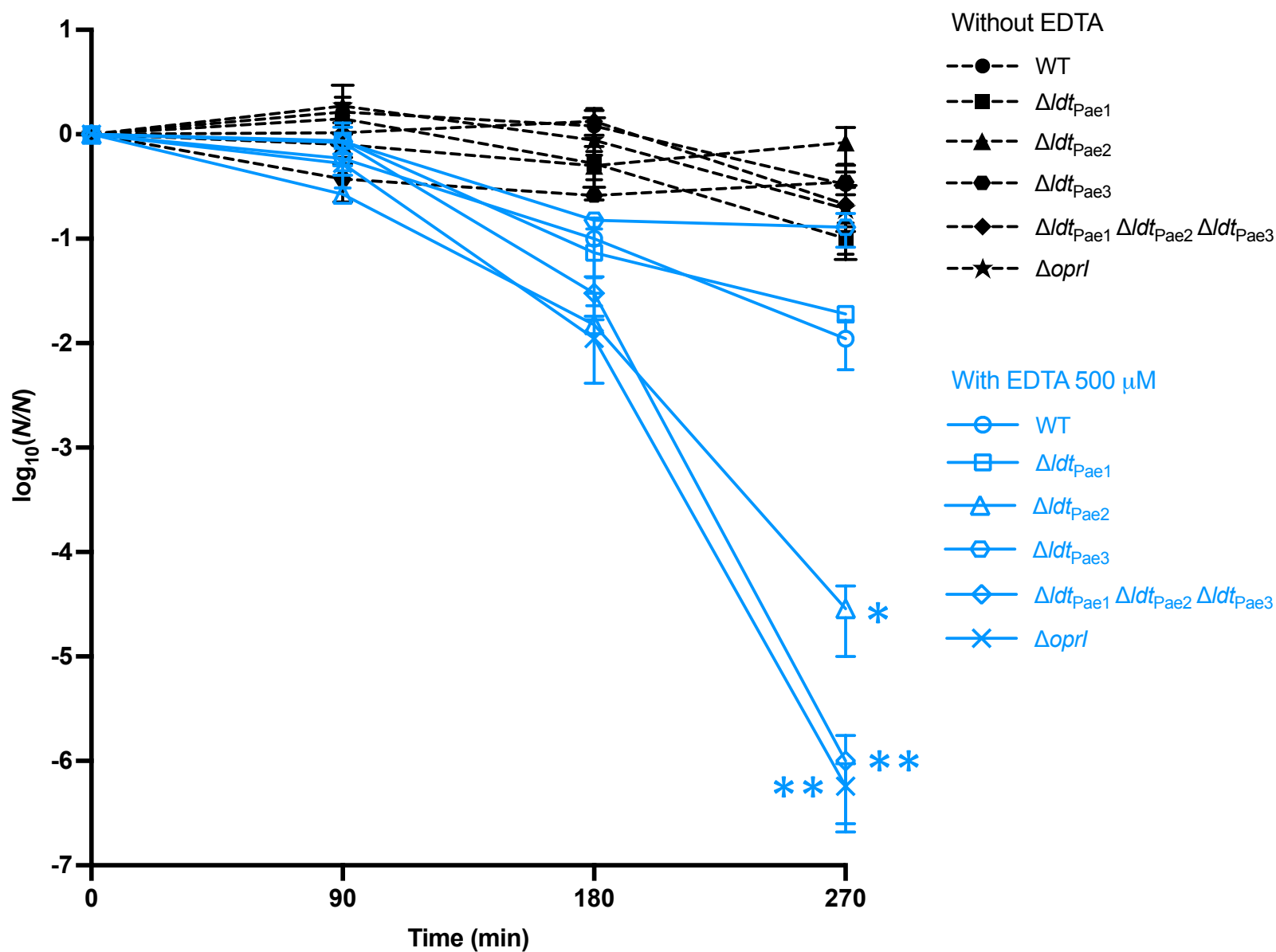
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M _{cal}	870.371	941.408	1,154.570	1,722.731	1,793.768	1,793.768	1,864.805

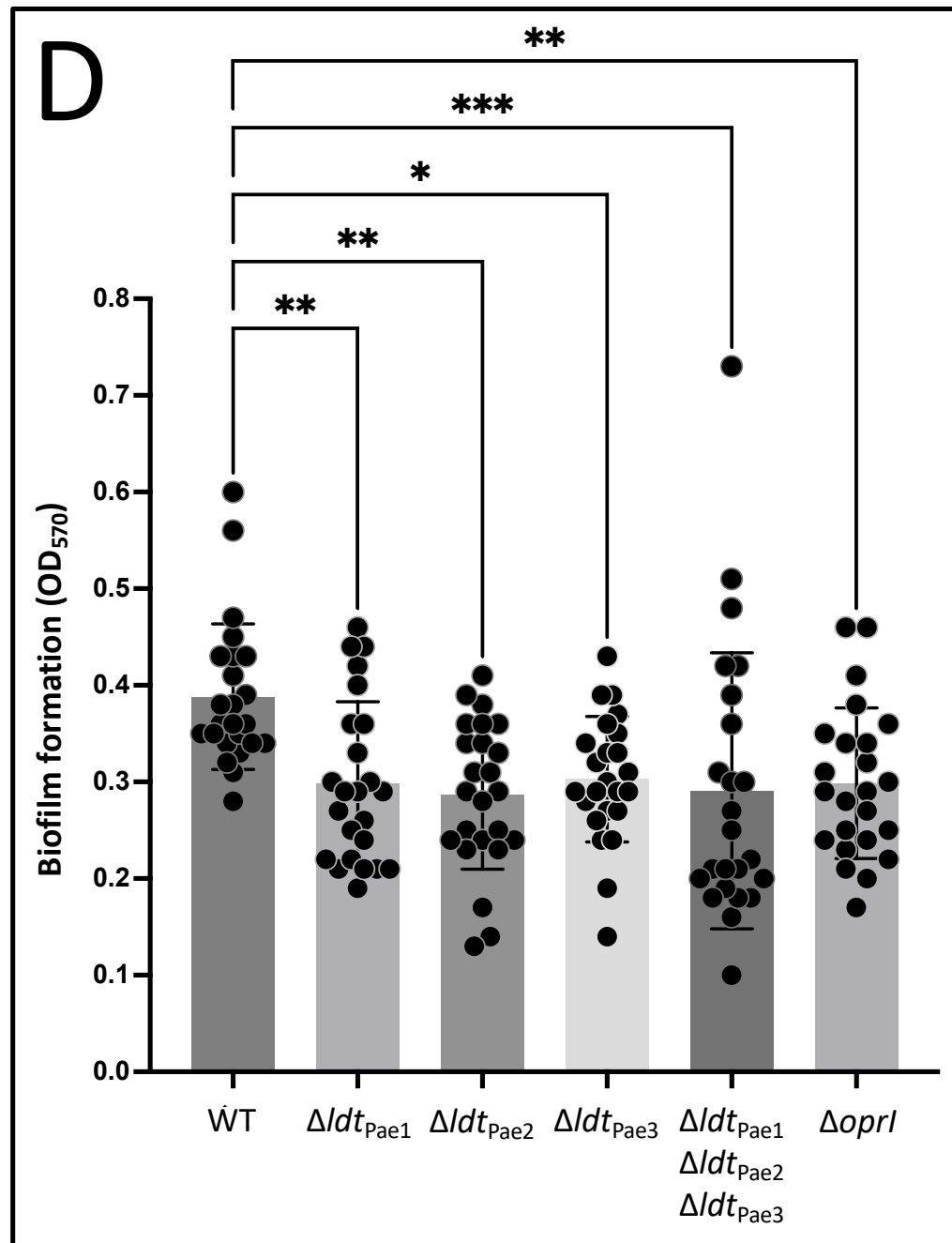
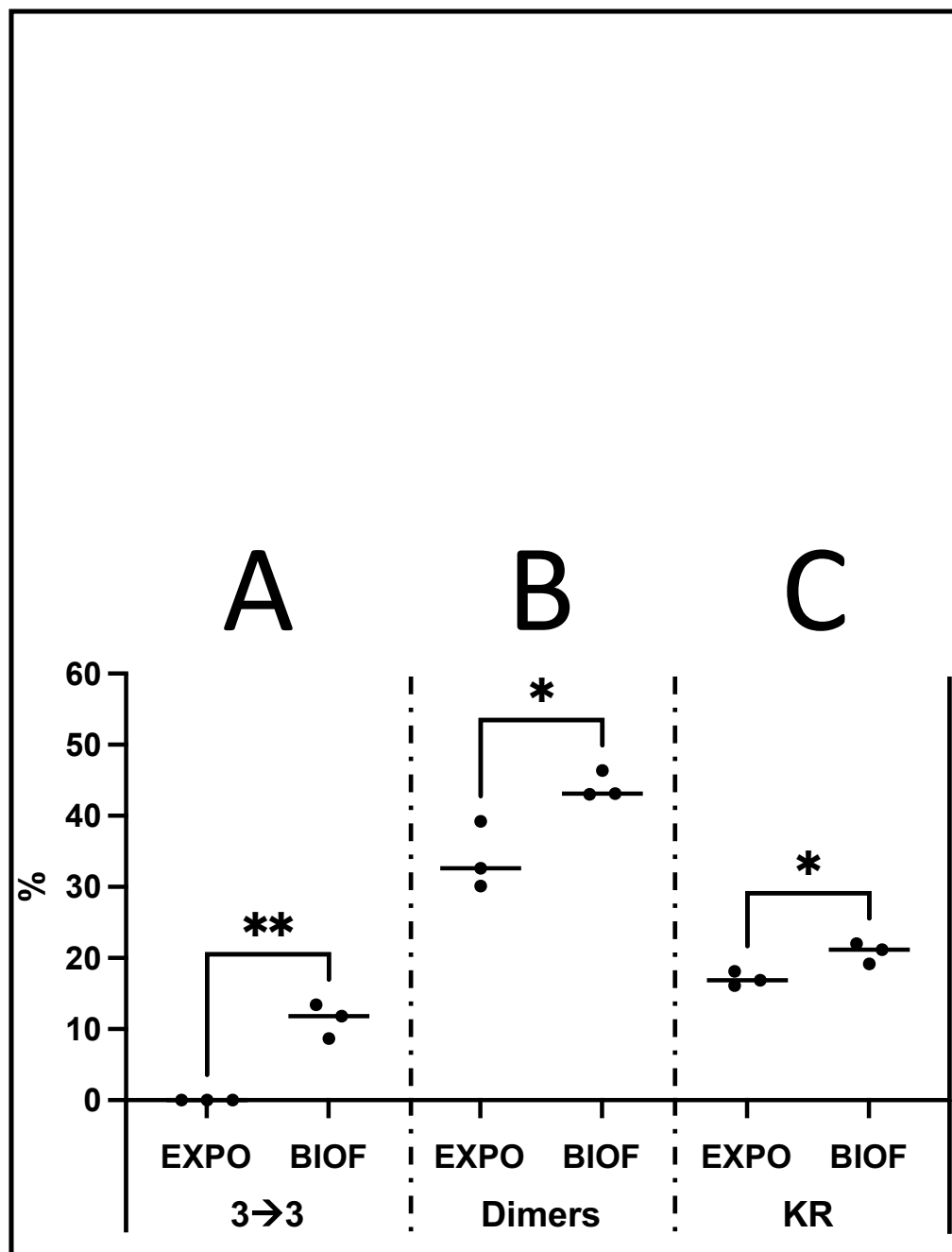




B Products of the reactions catalyzed by purified Ldt_{Pae1}

Substrate	Product	Mass	
		Calculated	Observed
Peptidoglycan fragment			
Tetra	Tri→Tetra	1,793.77	1,793.82
Lactoyl-Tetra	Lactoyl-Tri→ Lactoyl-Tetra	977.42	977.52
Tetra + D-Met	Tetra ending in D-Met	1,001.41	1,001.39
Lactoyl-Tetra + D-Met	Lactoyl-Tetra ending in D-Met	593.24	593.32
β-lactam			
Ampicillin	None	NA	NA
Ceftriaxone	Acylenzyme	36,698.7	36,699.2
Meropenem	Acylenzyme	36,686.5	36,686.9

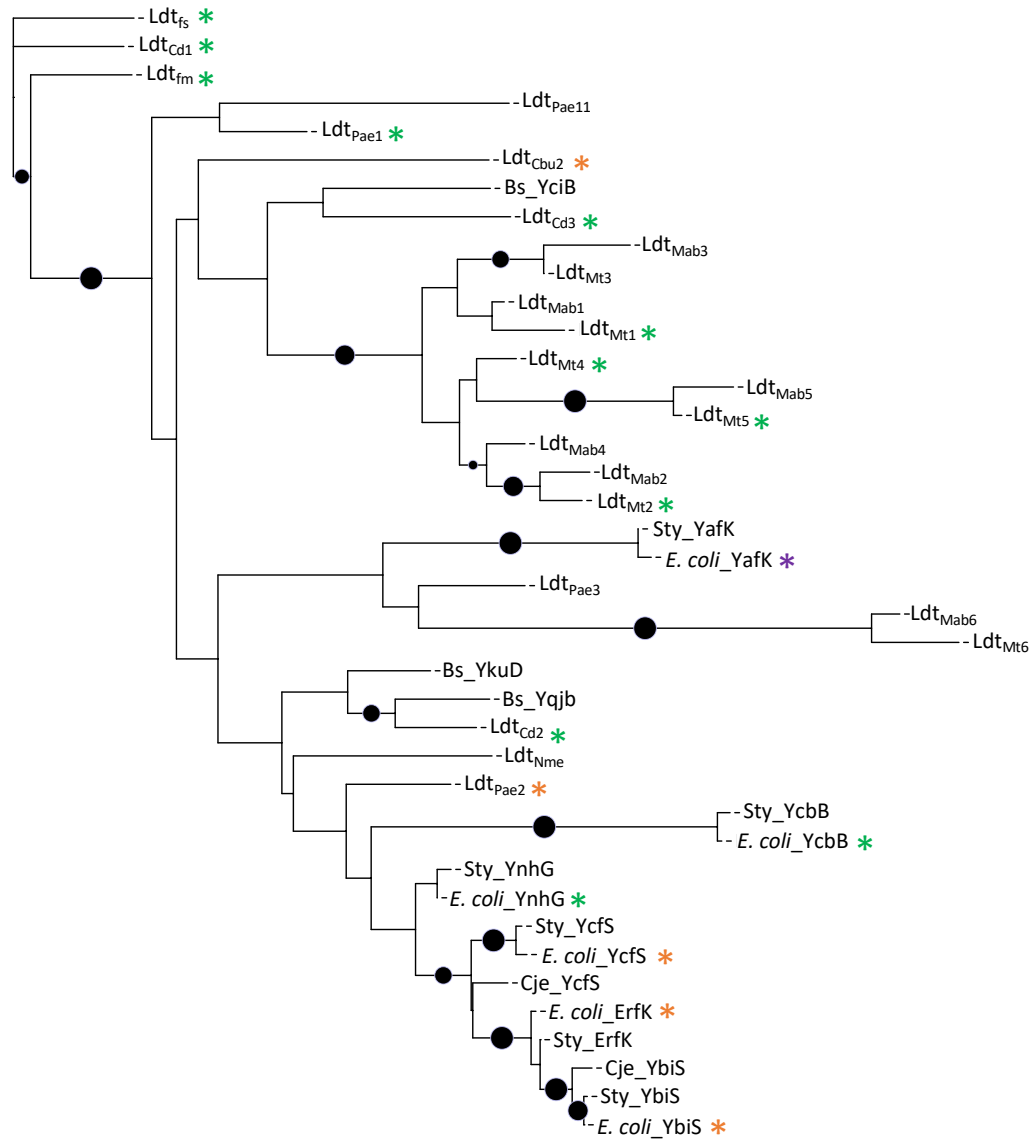




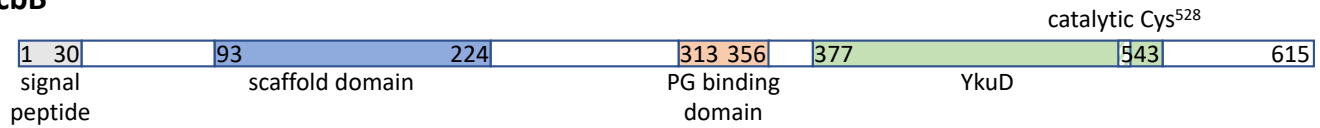
<i>E. coli</i> Lpp	MKAT-KLVLGAVILGSTLLA	GCSS	NAKIDQ--LS--SDVQTLN-AKVDQLSNDV	NAMRSDVQAAKDDAARANQRLDNMATKY-RK
<i>P. aeruginosa</i> OprI	MNNVLKFS--ALALAAVLAT	GCSS	HSKETEARLTATEDAAARAQARA	DEAYRKADEALGAAQKAQQTADEANERALRMLEKASRK

E.coli_YcbB	---SEAIYLHDTPNHNLF-----KRDTRLALSSGCVRVNKAASDLA--NMLLQ-----	167
Sty_ycbB	---SDAIYLHDTPNHNTLF-----QRDARALSSGCVRVNKASELA--NMLLQ-----	168
E.coli_YafK	GYEGKYLMIH-----GDCVSIIGCYAMTNQGIDEIFQFVTGALVFGQP	125
Sty_YafK	GYDGKYLMIH-----GACVSVGCGYAMTDSGIDEIFQFVTAALVFGQP	125
LdtPae3	--AGGMIMIHGTPLDDEYPE----WYFSTLDWTNGCIAMNNTDMREVWSVVKDGTLIEI-	135
LdtPae2	---VPGYLIHGSNKKFGI-----GTRTSHGCFRMYNADVTHLFSMISVGTSVRI-	136
E.coli_ErfK	---GRLYAIHGTNANFGI-----GLRVSQGCIRLRNDDIKYLFDNVPVGTRVQI-	135
Sty_erfK	---GRLYAIHGTNANFGI-----GLRVSQGCIRLRNDDIKYLFDPVVGTRVQI-	135
E.coli_YbiS	---GRLYAIHGTNANFGI-----GLRVSHGCVRLRNEDIKFLFEKVPVGTRV---	131
Sty_YbiS	---GRLYAIHGTNANFGI-----GLRVSHGCVRLRNDDIKFLFENVVPVGTRV---	131
Cje_YbiS	---GRLYAVHGTNANFGI-----GLRVSHGCVRLRDADIKWLYDNVPQGTTRVQFI	136
E.coli_YnhG	---GNGEYLIHGTSAPDSV-----GLRVSSGCI RMNAPDIKALFSSVRTGTVPKV-	136
Sty_YnhG	---GNGEYLIHGTSAPDSV-----GLRVSSGCI RMNAPDIKALFAQVRTGTVPKV-	136
E.coli_Ycfs	--YGGVYLLHGTNADFGI-----GMRVSSGCI RLRRDDDIKTLFSQVTPGTKVNI-	138
Sty_ycfs	--YGGVYLLHGTNADFGI-----GMRVSSGCI RLRDGDIETLFRQVTPGTKVNI-	138
Cje_ycfs	--GTGQYLIHGTNANFGI-----GMRVSSGCI RLRPDDIEALFNSVPKGTTRVQII	138
LdNme	--PKLGLGIHGTNAPASV-----PGVRSHGCVRMKSPDALEFAKTIASGSPASVI	133
Bs_YkuD	----QHYGIHGTNNPASI-----GKAVSKGCI RMHNKDVI ELASIVPNGTRVTIN	109
Ldtcd2	GTYGDTYGIHGNNNESSI-----GKHISGGCIRMHNKDVRWLFQVPVGSVDVII-	124
Bs_Yqjb	GTDGRIYGIHGTNREESV-----GKFVSNGCIRMHNDVHVHLFQTIPVGTRVLI-	124
LdtMt6	---VMGVNK----AKVPKGKSAFFHHTTDGGPTAGCVAIDDATLVQIIRWL RPGAVIAI-	178
LdtMab6	---VMGVNK----DRVPGGKSAFFVHSTDGGPTAGCVSLDDATLVKLIGWL RPGAVI---	158
Ldtfs	---VTQIGIHDS DHKLDKYGDKEAFK--TDAGSNGCINTPGTEVSKIFDVS YDGMPIII-	131
Ldtcd1	----GNIGIH DATWRNSFGGS---IY--KSSGSHGCVNTPFSKAKKIYENIEPGTPII--	107
Ldtfm	----SGVG IHDSDWQPEYGGD---LW--KTRGSHGCINTPPSVMKELFGMVEKGT PVLVF	126
LdtPae11	---WSVVQVSN-P---GQGL-----TPSEQLGKIRMDPTFVREMLGAMDVGSTLV--	99
LdtCbu2	---SGNAIHGSPYI-----S-----NRNTSHGCIRVYPGAAAWLSHYFMRAGTKV--	132
Bs_YciB	-----IKTE-----AEKL-----GTKASHGCIRLTIPDAKWVYENIPEHTKVVIS	126
LdtPae1	---WDGIALHA----GNLP-----GYPASHGCI RLPMFAKKLYGITGFSSTTVII	111
Ldtcd3	-----LYDSTGSYIIDGRL-----GEALSHGCIRLSTENAKWIYDNIPDTTTVII-	120
LdtMt5	---NNGEFIHANPMSAGAQG-----NSNVTNGCINLSTENAEQYRS AVYGD PVEV-	122
LdtMab5	---NNGEFIHANPNTIGQQG-----NTNVTNGCINLSLGAESYFRTAIYGD PVEV-	121
LdtMt3	---SRGLYVHSAPWALPALG-----LENVSHGCISLSREDAEWYNAVDIGDPVIV-	111
LdtMab3	---TRGLFVHSAPWAVPAMG-----YENVSHGCISLPPAAAEWYFNNVNI GDPVVV-	103
LdtMt1	---WSGVYVHSAPWSVNSQG-----YANVSHGCINLSPDNAAWYFDAVTVGDP IEV-	125
LdtMab1	---WGGVYVHSAPWSTGAQG-----NSNVSHGCINLSPDNASWYNTV SIGDP III-	116
LdtMt2	---YSGV FVHSAPWSVGAQG-----HTNTSHGCLNVSPSNAQWFYDHVKRGDIVEV-	126
LdtMab2	---YSGIYVHAAPWSVGAQG-----RTNTSHGCLNVSTANAKWFYENTKRGDVVIV-	121
LdtMt4	---NSGNFVHSAPWSVADQG-----KRNVT HGCINLSPANAKWFYDNFGSGDPVVV-	128
LdtMab4	---NSGIFVHAAPWSVGAQG-----KSDTSHGCINVN TDNATWFFNQSHPGDPVIV-	124

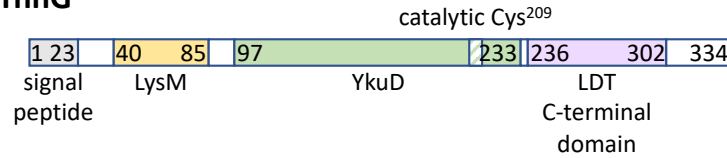
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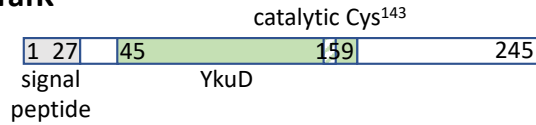
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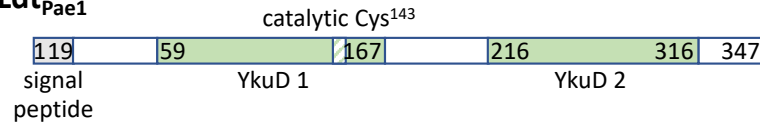
YnhG



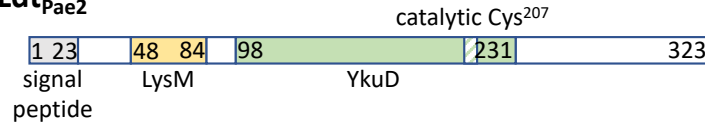
YafK



Ldt_{Pae1}



Ldt_{Pae2}



Ldt_{Pae3}

