



# Cleavage of Braun's lipoprotein Lpp from the bacterial peptidoglycan by a paralog of L,D-transpeptidases, LdtF

Raj Bahadur<sup>a</sup>, Pavan Kumar Chodiseti<sup>a</sup>, and Manjula Reddy<sup>a,1</sup>

<sup>a</sup>Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, 500007 Hyderabad, India

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The gram-negative bacterial cell envelope is made up of an outer membrane (OM), an inner membrane (IM) that surrounds the cytoplasm, and a periplasmic space between the two membranes containing peptidoglycan (PG or murein). PG is an elastic polymer that forms a mesh-like sacculus around the IM, protecting cells from turgor and environmental stress conditions. In several bacteria, including *Escherichia coli*, the OM is tethered to PG by an abundant OM lipoprotein, Lpp (or Braun's lipoprotein), that functions to maintain the structural and functional integrity of the cell envelope. Since its discovery, Lpp has been studied extensively, and although L,D-transpeptidases, the enzymes that catalyze the formation of PG–Lpp linkages, have been earlier identified, it is not known how these linkages are modulated. Here, using genetic and biochemical approaches, we show that LdtF (formerly *yafK*), a newly identified paralog of L,D-transpeptidases in *E. coli*, is a murein hydrolytic enzyme that catalyzes cleavage of Lpp from the PG sacculus. LdtF also exhibits glycine-specific carboxypeptidase activity on mucopeptides containing a terminal glycine residue. LdtF was earlier presumed to be an L,D-transpeptidase; however, our results show that it is indeed an L,D-endopeptidase that hydrolyzes the products generated by the L,D-transpeptidases. To summarize, this study describes the discovery of a murein endopeptidase with a hitherto unknown catalytic specificity that removes the PG–Lpp cross-links, suggesting a role for LdtF in the regulation of PG–OM linkages to maintain the structural integrity of the bacterial cell envelope.

bacterial cell wall | peptidoglycan | Lpp | LdtF

The gram-negative bacterial cell envelope is made up of an outer membrane (OM), an asymmetric bilayered lipid membrane which is surface exposed, and an inner membrane (IM) consisting of a phospholipid bilayer surrounding the cytoplasm. In between these two membranes is the periplasmic space in which a sac-like molecule, the peptidoglycan (PG or murein), is located (1). PG is an elastic heteropolymer that protects bacterial cells from lysis by internal osmotic pressure and from external stress conditions. It is a single, large macromolecule made up of multiple linear glycan strands that are interconnected with each other by short peptide chains, forming a net-like sacculus around the cytoplasmic membrane (Fig. 1). The glycan strands are polymers of alternating  $\beta$ -1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid (MurNAc) disaccharide units, in which the D-lactoyl moiety of each MurNAc residue is covalently attached to the first amino acid of the stem peptide. Normally, the peptide chains are of two to five amino acids with a pentapeptide made up of L-alanine (L-ala<sup>1</sup>)–D-glutamic acid (D-glu<sup>2</sup>)–mesodiaminopimelic acid (mDAP<sup>3</sup>)–D-ala<sup>4</sup>–D-ala<sup>5</sup> residues. In *Escherichia coli*, ~40% of the neighboring peptide chains are linked to each other, either between the D-ala<sup>4</sup> and mDAP<sup>3</sup> (D-ala–mDAP or 4–3) or two mDAP<sup>3</sup> residues (mDAP–mDAP or 3–3). Of these, the 4–3 cross-links are more prevalent and are formed by D,D-transpeptidases, whereas 3–3 cross-links are much less abundant and are catalyzed by L,D-transpeptidases (LDTs) LdtD and LdtE (2–4).

In several gram-negative bacteria, including *E. coli*, the OM is stapled to PG by a lipoprotein, Lpp or Braun's lipoprotein. Lpp is the first OM lipoprotein to be discovered almost five decades ago and has been studied extensively (5–9). It is a small protein

of 58 amino acids, which is numerically the most abundant (~10<sup>6</sup> molecules per cell), and is known to exist in two conformations, each occupying a distinct subcellular location in the cell envelope (9–11). One-third of total Lpp is in the periplasm covalently attached to the mDAP<sup>3</sup> residues of the PG peptides (bound form), whereas the other two-thirds spans the OM and is surface exposed (free form) (Fig. 1). The OM–PG tethering by Lpp has been shown to determine the width of the periplasm by controlling the IM–OM distance and to contribute to the stiffness of the cell envelope (12, 13). Although Lpp is not essential for the viability of *E. coli*, mutants that lack Lpp show several pleiotropic defects, such as increased sensitivity to hydrophobic agents, leakage of periplasmic contents, OM blebbing, vesiculation, cell separation defects, and deficiency in virulence, highlighting the role of Lpp in the maintenance of envelope integrity (8, 9, 14).

Three redundant LDTs, LdtA, LdtB, and LdtC, catalyze the formation of PG–Lpp cross-links by covalently attaching the extreme C-terminal residue of Lpp, lysine to the mDAP<sup>3</sup> residue of a tetrapeptide in the mature PG sacculus (15). In this reaction, the terminal D-ala residue of the tetrapeptide is lost, leading to the formation of a tripeptide–Lpp cross-link (Fig. 1). About 10% of the peptides in a PG sacculus are attached to Lpp, and this frequency is presumed to vary during conditions of stress (2, 5, 16–18).

*E. coli* encodes six LDTs, LdtA to LdtF, belonging to the YkuD family of proteins (3, 18–20). Of these, LdtA, LdtB, and LdtC catalyze the covalent attachment of Lpp to the PG; though these are redundant, LdtB is shown to be physiologically relevant because deleting *ldtB* alone abrogates the attachment of Lpp to a significant extent (15). Ribosome profiling (10) has also shown

## Significance

Bacterial cell walls contain a protective exoskeleton, peptidoglycan, which is a target of several clinically important antimicrobials. In gram-negative bacteria, peptidoglycan is covered by an additional lipid layer, outer membrane, that serves as a permeability barrier against the entry of toxic molecules. In some bacteria, an abundant lipoprotein, Lpp, staples the outer membrane to peptidoglycan to maintain the structural integrity of the cell envelope. In this study, we identify a previously unknown hydrolytic enzyme that cleaves Lpp from the peptidoglycan and show how the outer membrane–peptidoglycan linkages are modulated in *Escherichia coli*. Overall, this study helps in understanding the fundamental bacterial cell wall biology and in the identification of alternate drug targets for the development of new antimicrobials.

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The authors declare no competing interest.

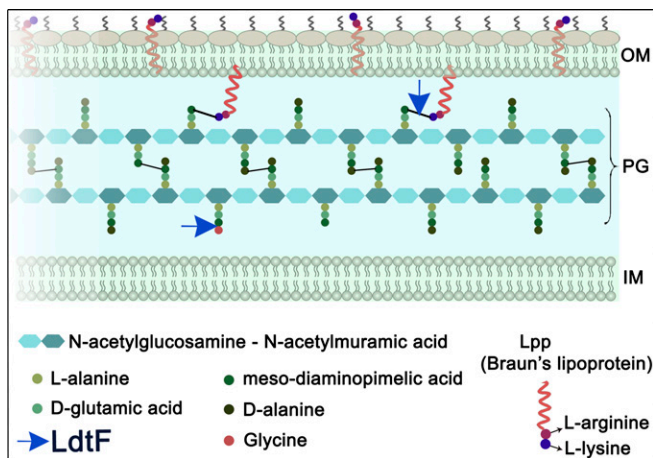
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<sup>1</sup>To whom correspondence may be addressed. Email: manjula@cmb.res.in.

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**Fig. 1.** Diagrammatic representation of the cell envelope of *E. coli*. The cell envelope consists of three layers: OM, PG, and IM. PG is stapled to the OM by Lpp or Braun's lipoprotein (red helix), which exists in either bound or free form (5–9). In the bound form, the N-terminal end of Lpp is anchored to the OM, whereas the C-terminal lysine residue (purple circle) is covalently attached to an mDAP residue (dark green) of the PG stem peptides. The free form of Lpp spans the OM and is exposed to the surface (11). LdtF is identified in this study as an endopeptidase that cleaves the PG–Lpp cross-links and as a glycine-specific carboxypeptidase.

the abundance of LdtB to be much higher (~5,000 copies per cell) than LdtA and LdtC (~50 and 500, respectively). On the other hand, the 3–3 cross-link formation in the PG sacculus is catalyzed by LdtD and LdtE (21).

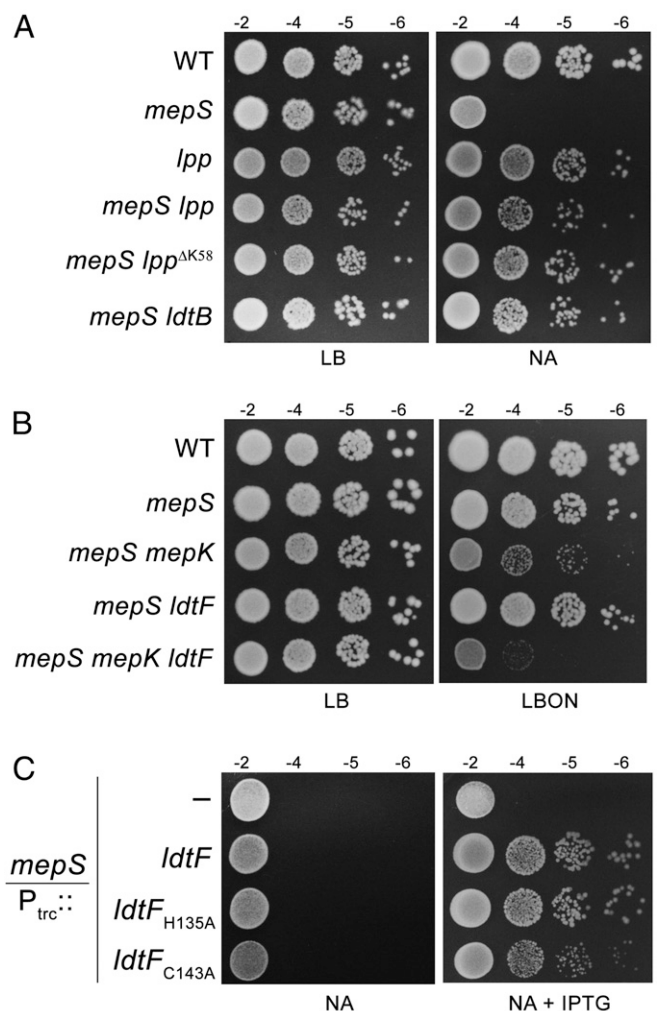
Apart from their ability to form cross-links, LdtA through LdtE catalyze an amino acid exchange reaction in the periplasm, wherein the terminal D-al<sup>4/5</sup> residue of the stem peptides is substituted with either glycine or a variety of noncanonical D-amino acids (NCDAA), such as D-tryptophan, D-methionine, or D-aspartate (2, 21, 22). The significance of this exchange in *E. coli* is not clear, although it is believed that these substituted muropeptides do not participate in further steps of PG polymerization. LDTs are presumed to have a larger role in the maintenance of structural integrity of PG because of their ability to form cross-links de novo in a mature PG sacculus independent of active PG precursor synthesis (18).

LdtF is a recently identified paralog of LDTs and is implicated in facilitating the formation of 3–3 cross-links; however, its precise function remains unclear (18–20). Here, we show that LdtF (encoded by *yafK*) is a murein L<sub>D</sub>-endopeptidase that cleaves Lpp from the PG sacculus. We initially identified *ldtF* because of its genetic interaction with *mepS*, a gene encoding a major PG elongation-specific D,D-endopeptidase (23). Further genetic and biochemical analysis demonstrated the role of LdtF in hydrolyzing the products generated by the activity of other LDTs. LdtF cleaves Lpp, which is bound to the PG sacculus, and, in addition, cleaves the terminal glycine residue that is incorporated into the stem peptides due to the periplasmic exchange reaction of LDTs. However, LdtF was not able to cleave the terminal NCDAA residues from the muropeptides. To summarize, this study identifies a murein endopeptidase with a previously unknown catalytic specificity having an ability to modulate the Lpp-mediated OM–PG linkages.

## Results

**Absence of PG–Lpp Linkages Confer Growth Advantage to an *E. coli* Mutant Lacking an Elongation-Specific D,D-Endopeptidase, *mepS*.** We showed earlier that the absence of 3–3 cross-link-forming LDTs (*ldtD* and *ldtE*) confers a growth advantage to a mutant lacking an elongation-specific 4–3 cross-link-cleaving D,D-endopeptidase,

*MepS*, signifying the importance of cleavage of both 4–3 and 3–3 cross-links to make space for incorporation of new PG material during cell elongation (23, 24). To examine whether the tethering of OM to PG by Lpp also affects the process of PG expansion, we introduced a deletion of Lpp into a mutant lacking *MepS* and examined the growth phenotypes of the double mutant on nutrient agar (NA) because the *mepS* deletion mutant is unable to grow on NA at high temperatures (25). Fig. 2A shows that the absence of Lpp restores a moderate amount of growth to the *mepS* deletion mutant on NA. In addition, a mutant *lpp* allele that lacks the C-terminal lysine residue and hence is unable to bind PG (Lpp<sup>ΔK58</sup>; ref. 11) confers suppression like that of Lpp deletion. Likewise, deletion of LdtB, which catalyzes the formation of mDAP–Lpp linkages, conferred growth to the *mepS* deletion mutant (Fig. 2A and *SI Appendix*, Fig. S1A and S2). However, deletion of LdtA or LdtC, which also link Lpp to mDAP, did not have any effect on *mepS* growth (*SI Appendix*, Fig. S1A). Surprisingly, deletion of LdtF, the newly identified paralog of LDTs, conferred a very small yet consistent growth defect to the *mepS* single mutant (*SI Appendix*, Fig. S1B), which was further exacerbated in an *mepS*



**Fig. 2.** Genetic interactions of *mepS* with LDTs. (A) WT (MG1655) and its mutant derivatives carrying either deletion of *lpp*, *ldtB*, or *lpp*<sup>ΔK58</sup>, an allele lacking C-terminal lysine residue, were tested for viability on NA at 37 °C. (B) Indicated strains were grown and viability was tested on LBON agar plates at 37 °C. (C) An *mepS* deletion mutant carrying either vector pTrc99a (P<sub>trc</sub>::), pRB1 (P<sub>trc</sub>::*ldtF*), pRB2 (P<sub>trc</sub>::*ldtF*<sub>H135A</sub>), or pRB3 (P<sub>trc</sub>::*ldtF*<sub>C143A</sub>) were tested for viability on NA at 37 °C with or without IPTG (0.25 mM).

mutant lacking *mepK*, a gene encoding the 3–3 cross-link–cleaving PG hydrolase (Fig. 2B).

As these results intrigued us, we further investigated the role of LdtF by introducing the plasmids encoding each of the LDTs (26) into the *mepS* mutant. As shown in *SI Appendix*, Fig. S3, plasmids encoding *ldtA*, *ldtB*, *ldtC*, *ldtD*, and *ldtE* did not confer growth to the *mepS* mutant, whereas a plasmid encoding *ldtF* alone was able to moderately suppress the growth defects of *mepS* mutant. Another plasmid derivative carrying cloned *ldtF* downstream to an IPTG-dependent promoter ( $P_{trc}::ldtF$ ) also suppressed the growth defects of the *mepS* mutant on NA (Fig. 2C), indicating that LdtF may have a distinct function compared to that of other LDTs. LdtF belongs to the YkuD family of proteins, and members of this family contain the LDT domain with an invariant cysteine residue at the active site (27). To further validate the role of LdtF, we constructed a mutant derivative with an alanine residue substituted for cysteine (LdtF-C143A) and examined its ability to suppress the *mepS* mutation. Fig. 2C shows that a plasmid encoding LdtF-C143A poorly suppressed the *mepS* deletion mutant, whereas another variant coding for LdtF-H135A behaved like that of wild type (WT). However, deletion of *ldtF* alone in a WT strain did not confer any discernible phenotype when grown on Luria–Bertani (LB), LB broth without NaCl (LBON), or NA plates at 30, 37, or 42 °C, except for a slight reduction in the doubling time during the exponential phase of growth (*SI Appendix*, Fig. S4A). In addition, the *ldtF* deletion mutant did not exhibit significant sensitivity to any of the cell wall antibiotics, such as cephalixin, cefsulodin, mecillinam, or vancomycin (*SI Appendix*, Fig. S4B).

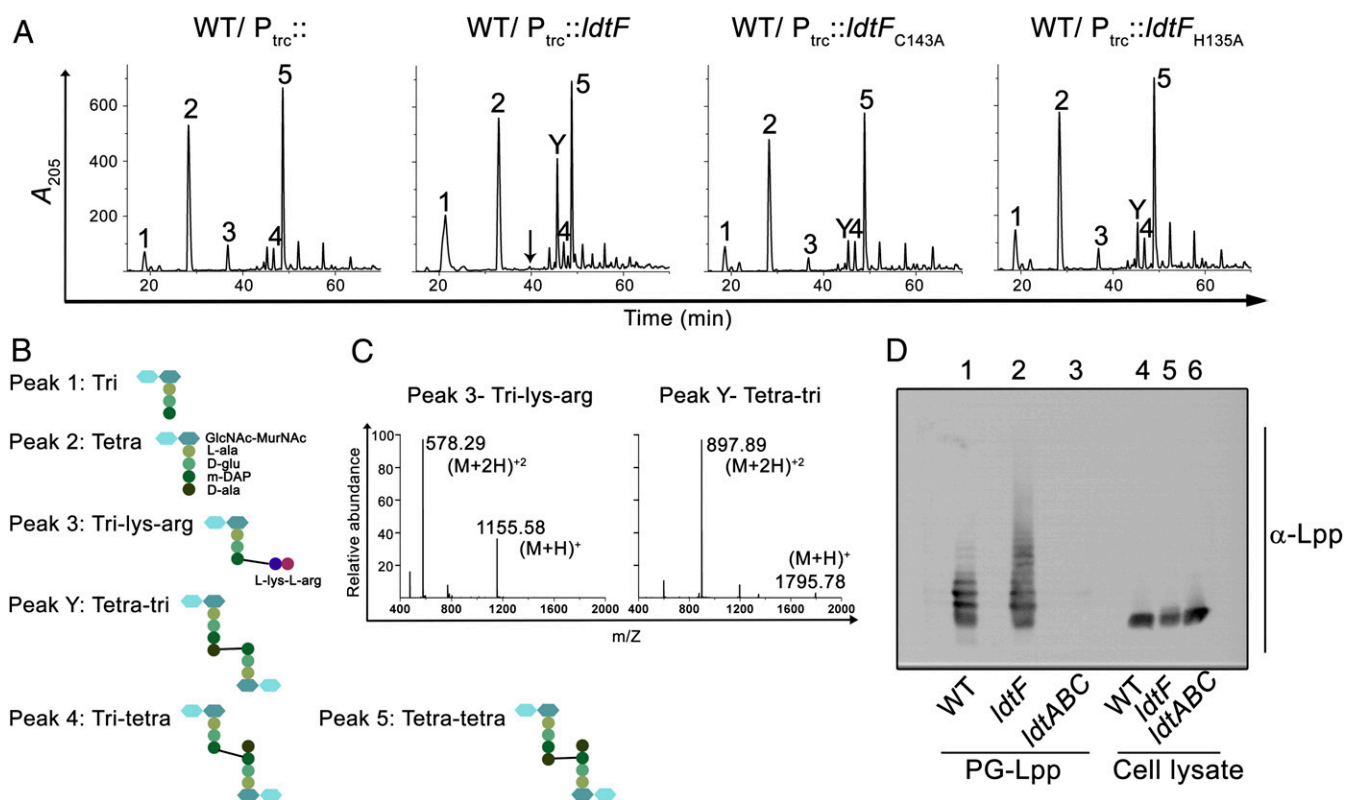
**LdtF Modulates PG–Lpp Linkages In Vivo.** To understand the basis of LdtF's function, we examined the composition of PG in strains either having a deletion or multiple copies of *ldtF*. PG sacculi from these strains were prepared and digested with a muramidase (mutanolysin), followed by separation of soluble muropeptides by reverse-phase-high-performance liquid chromatography (RP-HPLC) and identification of the peaks by mass spectrometry (MS) or MS–MS analysis. No major difference was observed in the muropeptide profile of the *ldtF* deletion derivative compared to that of WT (*SI Appendix*, Fig. S5). In contrast, the PG sacculi of cells carrying additional copies of *ldtF* had considerable alterations (Fig. 3A and *SI Appendix*, Table S3), the most significant being the absence of peak 3 (tri-lys-arg), which is a disaccharide tripeptide attached to a lys-arg dipeptide (Fig. 3B and C). Tri-lys-arg muropeptides are generated because of the proteolytic activity of pronase, which is used during preparation of PG sacculi to remove bound Lpp. Pronase cleaves Lpp at the 56th position, leaving the extreme C-terminal lys-arg dipeptide attached to the mDAP residue of the stem peptides, resulting in the generation of several species of muropeptides bound to the lys-arg dipeptide (2). In addition to the absence of peak 3, a muropeptide peak eluting at 46 min (labeled “Y”) was significantly elevated in the PG sacculi of cells carrying additional copies of LdtF (Fig. 3A). MS–MS analysis indicated this peak to be a tetra-tri dimer linked by a 4–3 cross-bridge with a molecular mass of 1,794 Da (Fig. 3B and C and *SI Appendix*, Fig. S6). The absence of peak 3 with a concomitant increase in peak 1 (a monomer of tri) allowed us to speculate that LdtF may have an ability to modulate the mDAP–Lpp linkages. Though the source of peak Y is not clear, it was not detected in a strain deleted for Lpp, suggesting it may have originated by the activity of LdtF on PG–Lpp cross-links (*SI Appendix*, Fig. S7A). Additionally, the incidence of peak Y was not dependent on the presence of functional LdtD and LdtE (*SI Appendix*, Fig. S7B). Furthermore, all other alterations observed due to overexpression of LdtF disappeared in a strain lacking Lpp, reinforcing the suggestion that LdtF functions downstream of Lpp (*SI Appendix*, Fig. S7A). As shown in Fig. 3A, an analysis of PG in strains carrying plasmids encoding either LdtF-C143A or LdtF-H135A indicated a direct role for LdtF in modulation of mDAP–Lpp linkages (*SI Appendix*, Table S3).

Because the above results implicated LdtF in the regulation of PG–Lpp linkages, we examined the extent of these cross-links in cells lacking LdtF. To perform this experiment, Lpp-bound PG sacculi were prepared from WT and *ldtF* deletion mutant. Sacculi from both strains were digested with mutanolysin, soluble muropeptides were collected, and normalized amounts (*SI Appendix*, Fig. S8) were electrophoresed using SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) followed by Western blotting and detection with anti-Lpp antibody. Fig. 3D shows that the PG sacculi derived from the LdtF deletion mutant indeed contain a greater abundance of high-molecular-weight Lpp-bound muropeptides compared to that of WT; however, the level of low-molecular-weight Lpp-bound muropeptides were unchanged. This observation suggested an interesting possibility of LdtF specifically modulating the larger oligomeric Lpp–cross-linked muropeptides of the PG sacculus, and the implications of this result are further discussed below.

**LdtF Is a Murein Endopeptidase that Cleaves PG–Lpp Linkages.** To examine the enzymatic activity of LdtF, a signal-less, hexahistidine-tagged LdtF (LdtF<sup>20–246</sup>-His<sub>6</sub>) was overexpressed and purified. Treatment of soluble muropeptides derived from the PG sacculi of WT *E. coli* with purified LdtF yielded muropeptide fraction that totally lacked tri-lys-arg (peak 3) and tetra-tri-lys-arg (peak 6) with a concomitant increase in tri- and tetra-tri muropeptides (Fig. 4A). Cleavage of tri-lys-arg and tetra-tri-lys-arg into tri- or tetra-tri muropeptides was also confirmed using purified fractions (Fig. 4B–D).

We next examined the ability of LdtF to cleave the bound Lpp from the intact PG sacculi. To perform this experiment, Lpp-bound PG sacculi from WT and *ldtF* mutant were isolated, and equal amounts of each were treated with purified LdtF. The soluble fraction was electrophoresed on SDS-PAGE, and Lpp was detected by Western blot analysis using anti-Lpp antibody. As a positive control, PG sacculi treated with pronase were used. Fig. 4E shows that both LdtF and pronase cleave Lpp from the PG sacculi and that the amount of Lpp released from the sacculi of LdtF deletion mutant was considerably higher (approximately fivefold) than that of the WT (compare lanes 2 and 3 with 5 and 6). The remaining insoluble PG fraction was further analyzed by RP-HPLC, and, as expected, the lys-arg muropeptides were not detected in LdtF-treated PG, whereas pronase-treated PG contained the lys-arg muropeptides (*SI Appendix*, Fig. S9). Overall, these results demonstrate the catalytic specificity of LdtF on PG–Lpp or PG–lys-arg substrates.

**LdtF Is a Glycine-Specific Carboxypeptidase that Cleaves Terminal Glycine Residue from the Stem Peptides.** The above experiments clearly demonstrated hydrolytic activity of LdtF on PG–Lpp linkages formed by LdtA, LdtB, and LdtC. LDTs also exchange the terminal D-ala of stem peptides with a glycine residue. To examine whether LdtF has any activity on glycine-substituted muropeptides, soluble muropeptides of a WT strain grown with glycine supplementation were used as substrates for LdtF. As expected, growth of WT *E. coli* with exogenously added glycine resulted in the accumulation of a large number of muropeptides with glycine at position four, whose identity is determined by MS analysis (*SI Appendix*, Fig. S10). Fig. 5A shows that LdtF effectively removes glycine from a variety of glycine-containing muropeptides. LdtF also cleaved several glycine-containing muropeptides prepared from PG sacculi of a strain overexpressing LdtD (Fig. 5B). Of these, three distinct types of glycine-containing muropeptides were purified to homogeneity, and Fig. 5C–E show that LdtF removes glycine residue from all these muropeptides. However, the abundance of glycine-containing muropeptides remained the same in both WT and *ldtF* deletion mutant when grown with glycine supplementation, suggesting the existence of alternate carboxypeptidases that cleave the terminal glycine residue. In support of this idea, the *ldtF* deletion mutant was



**Fig. 3.** LdtF modulates PG-Lpp linkages. (A) HPLC chromatograms of PG sacculi of WT carrying either vector ( $P_{trc}::$ ), pRB3 ( $P_{trc}::ldtF$ ), pRB2 ( $P_{trc}::ldtF_{H135A}$ ), or pRB3 ( $P_{trc}::ldtF_{C143A}$ ). Cultures were grown to an  $A_{600}$  of  $\sim 1$  in LB containing 0.2 mM IPTG for PG isolation. The black arrow indicates the absence of tri-lys-arg peak. (B) Structures of muropeptides. (C) Mass spectra of peaks 3 and Y, showing molecular mass ( $M+H$ ) $^+$  of 1,155.58 and 1,795.78 Da. (D) Determination of PG-Lpp linkages in the WT and  $ldtF$  mutant was done by treating the intact sacculi with mutanolysin, followed by electrophoresis of the soluble muropeptides. Lpp-containing muropeptides were visualized by Western blot using anti-Lpp antibody. PG from the  $ldtABC$  mutant was used as negative control. Cell lysates of WT,  $ldtF$ , and  $ldtABC$  were used as controls (lanes 4, 5, and 6).

not sensitive to the addition of glycine and behaved just like that of the WT strain.

Considering an earlier report that D,D-carboxypeptidases hydrolyze the terminal glycine from the stem peptides (2, 28), we made a quadruple mutant deleted for major D,D-carboxypeptidases, DacA, DacB, DacC, and DacD, and tested for sensitivity to glycine. As expected, the quadruple mutant formed smaller-sized colonies on glycine-supplemented media (SI Appendix, Fig. S11). The introduction of an  $ldtF$  deletion marginally exacerbated the defect of this quadruple mutant, whereas multiple copies of  $ldtF$  moderately improved the growth of this mutant on glycine-containing media (SI Appendix, Fig. S11), implicating a role for LdtF in the removal of terminal glycine residue from the stem peptides. In sum, the above results demonstrate that LdtF is a glycine-specific carboxypeptidase.

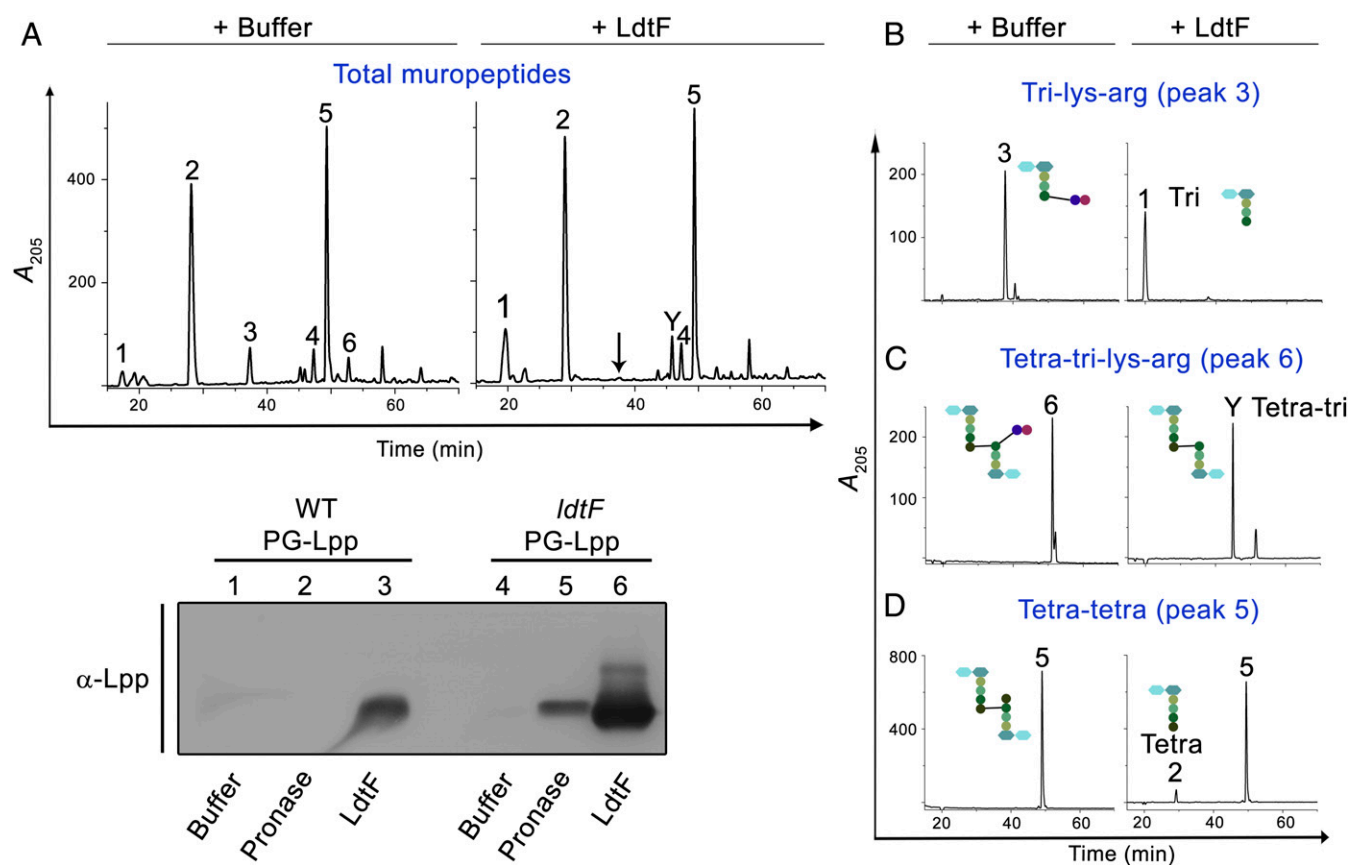
**LdtF Does Not Cleave NCDAA from the Stem Peptides.** To examine whether LdtF also cleaves the terminal NCDAA residues that are substituted by the exchange reaction of the LDTs, PG sacculi were made from WT *E. coli* grown in the presence of D-methionine, D-tryptophan, or D-phenylalanine (22). Soluble muropeptides of these PG sacculi were separated, and the peaks containing the NCDAA substitutions were identified by MS analysis (SI Appendix, Fig. S12A) and used as substrates for LdtF (SI Appendix, Fig. S12B). However, LdtF was not able to cleave the terminal NCDAA from any of these muropeptides (SI Appendix, Fig. S12B).

**LdtF Removes Lpp-Mediated IM-PG Linkages.** Lpp is transported from the cytosol into the periplasm by Sec-mediated pathway and is eventually translocated to the OM by lipoprotein translocating

machinery, LolABCDE (29). However, in certain transport-defective mutants, Lpp is stalled at the periplasmic face of the IM, leading to the formation of IM-PG linkages by LDTs (30, 31). It has been shown recently that the absence of two small cytoplasmic membrane proteins, DcrB and YciB, leads to mislocalization of Lpp at the IM, resulting in lethal IM-PG cross-links, and that this lethality is suppressed by the deletion of either Lpp or LdtB (31). To examine the ability of LdtF to cleave the Lpp bound to IM, we made use of this mutant and observed that a deletion of  $ldtF$  exacerbates the growth defect of the  $yciB$   $dcrB$  double mutant (Fig. 6A). In addition, the introduction of a multicopy  $ldtF$  plasmid ( $P_{trc}::ldtF$ ) partially restored the growth of the  $yciB$   $dcrB$  double mutant, suggesting that LdtF may also cleave Lpp bound to the IM (Fig. 6B).

## Discussion

Here, we report the identification of a previously unknown PG hydrolase, LdtF, that cleaves Lpp (or Braun's lipoprotein), an abundant OM lipoprotein that links OM to the PG sacculus in *E. coli*. LdtF is also a glycine-specific carboxypeptidase that removes the terminal glycine residue from the PG muropeptides. LdtF is a recently identified member of the YkuD family of proteins in *E. coli*; the other paralogs of this family comprise LdtA, LdtB, and LdtC, which catalyze the formation of mDAP-Lpp linkages, and LdtD and LdtE, which catalyze the formation of mDAP-mDAP cross-links. This study also represents an instance wherein members of a paralogous family perform contrasting but not overlapping functions. While this manuscript is in review, an



**Fig. 4.** Endopeptidase activity of LdtF. Soluble muropeptides of WT PG sacculi (A), purified tri-lys-arg (B), purified tetra-tri-lys-arg (C), or purified tetra-tetra dimer (D) were incubated either with buffer or LdtF (4  $\mu$ M) for 16 h and separated by RP-HPLC. LdtF cleaved peak 3 (tri-lys-arg) to yield tri (peak 1) and peak 6 (tetra-tri-lys-arg) to yield tetra-tri (peak Y). LdtF showed an extremely weak activity on tetra-tetra dimer (peak 5). (E) Cleavage of Lpp from the PG sacculi (containing bound Lpp) of WT or *ldtF* mutant was tested by incubating the PG sacculi either with buffer, pronase (0.2 mg/mL), or LdtF (4  $\mu$ M) for 16 h at 30  $^{\circ}$ C. Pronase, a nonspecific protease, is used as a positive control.

independent study by Winkle et al. (32) has also demonstrated identical biochemical activities for LdtF.

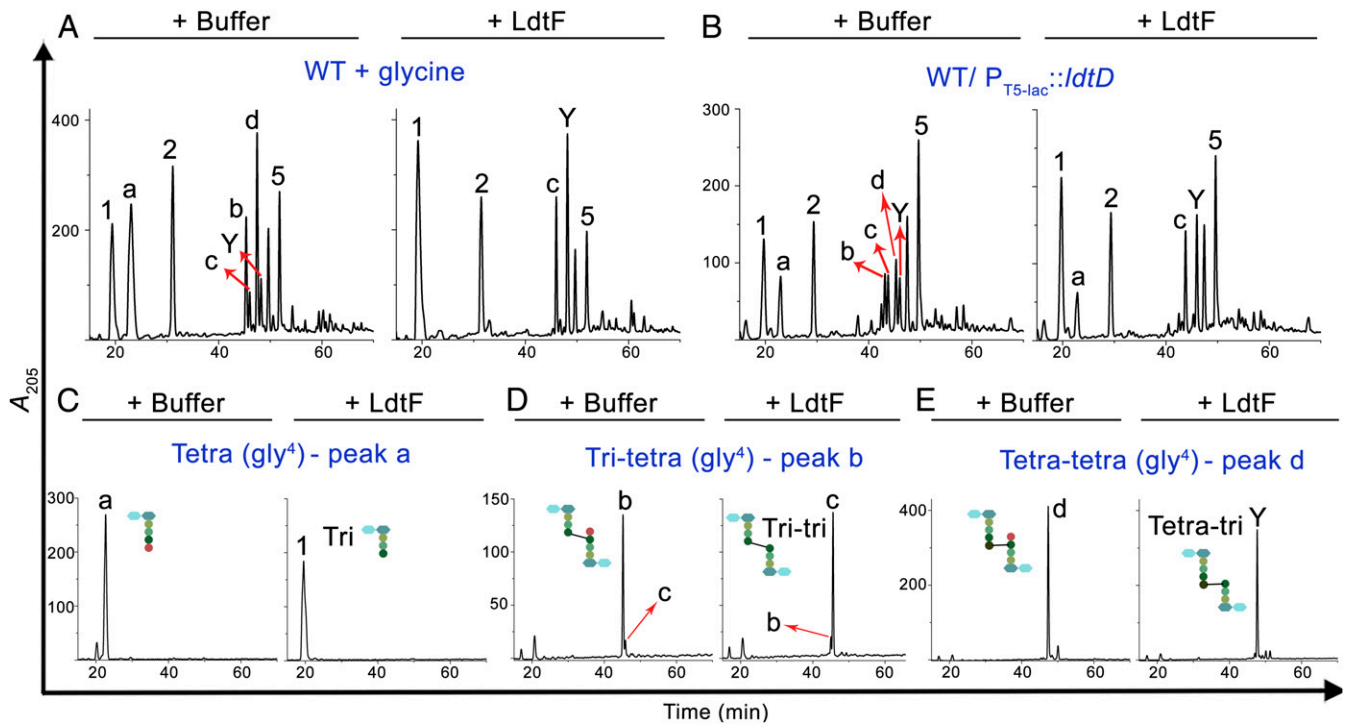
**Role of LdtF in Maintenance of Envelope Structure and Stability.** We identified LdtF in this study because its absence enhanced growth defects of a mutant lacking two of the PG elongation-specific endopeptidases, MepS and MepK, and, additionally, multiple copies of *ldtF* rescued the defects of *mepS* mutant (Fig. 2 B and C). Moreover, we observed that the absence of LdtF increases the PG-Lpp linkages (Fig. 3D), whereas more copies of *ldtF* decrease the level of PG-bound Lpp (Fig. 3A), suggesting a role for LdtF in modulating the degree of PG-Lpp cross-linkages. Subsequent biochemical analysis confirmed LdtF to be a hydrolase having two distinctive enzymatic functions—an L,D-endopeptidase activity that cleaves mDAP-Lpp cross-links and a carboxypeptidase activity that cleaves mDAP-gly linkages (Figs. 4 and 5). Interestingly, two other YkuD family proteins, Csd6 and Pgp2, function as L,D-carboxypeptidases (33, 34). These hydrolases, including LdtF, have a distinctive YkuD domain with either an alanine or asparagine residue occurring at the second position after the catalytic cysteine, whereas synthases such as LdtA to LdtE contain an arginine residue (32–34).

LdtF was earlier identified because a transposon insertion in *ldtF* (*yafK*) caused defective biofilm formation in an enteroaggregative *E. coli* strain (35). LdtF deletion has also been shown to confer additive sickness to a mutant defective in the transport of lipopolysaccharide (18). Although the basis of the above phenotypes is not clear, elevated OM-PG linkages may result in a defective cell

envelope, leading to such phenotypes. Excess OM-PG linkages may also alter the plasticity of the cell wall, resulting in decreased fitness and survival of *E. coli*. Nevertheless, under laboratory conditions, the absence of *ldtF* did not result in a large effect on the growth of *E. coli*, except for a small decrease in the doubling time (*SI Appendix*, Fig. S4).

It is interesting to note that although the abundance of lower-molecular-weight Lpp-bound muropeptides was comparable in both WT and *ldtF* mutant (Fig. 3D and *SI Appendix*, Fig. S5), the amount of bound Lpp is considerably higher in the absence of LdtF (Figs. 3D and 4E). The occurrence of larger Lpp-bound oligomeric muropeptides in the *ldtF* mutant (Fig. 3D) strongly suggests that LdtF may preferentially cleave PG-Lpp cross-links of higher-order structures in the PG sacculus. Lpp-mediated OM-PG cross-linkages resulting in the formation of large oligomers may distort the symmetry or the organization of the cell envelope, and LdtF may perhaps work toward eliminating such linkages. In addition, the effect of *ldtF* alleles on the growth of mutants in which Lpp is stalled at the IM (Fig. 6), suggests a role for LdtF in the removal of rare IM-PG linkages that may occur during transport of Lpp across the periplasm. LdtF may also facilitate PG turnover as the Lpp-linked muropeptides may not efficiently get recycled unless Lpp is cleaved from the PG sacculi.

Earlier studies implicated LdtF in facilitating the formation of 3–3 cross-links because ectopic expression of LdtF along with coexpression of LdtD or LdtE (in a strain lacking *ldtABCDEF*) increased the level of 3–3 cross-links (18–20). However, we did



**Fig. 5.** LdtF is a glycine-specific carboxypeptidase. Soluble muropeptides generated from WT cells grown in minimal A medium (36) supplemented with 50 mM glycine (A), soluble muropeptides of WT/*P*<sub>T5-lac</sub>::*ldtD* (B), purified tetra (gly<sup>4</sup>) (C), tri-tetra (gly<sup>4</sup>) (D), or tetra-tetra (gly<sup>4</sup>) (E) were incubated either with buffer or LdtF (4 μM) and processed as described above. LdtF cleaved the terminal glycine residue completely from peak “a” (tetra-gly<sup>4</sup>), “b” (tri-tetra-gly<sup>4</sup>), or “d” (tetra-tetra-gly<sup>4</sup>) to yield peak 1 (tri), “c” (tri-tri), or Y (tetra-tri).

not observe increased 3–3 dimers when LdtF was overexpressed in WT; in contrast, we detected increased 4–3 linked dimers (peak Y) whose occurrence was dependent on Lpp but not on LdtD or LdtE (*SI Appendix, Fig. S7*). The basis of this discrepancy is not clear—it may perhaps be due to the strain background used for these studies and needs to be further investigated.

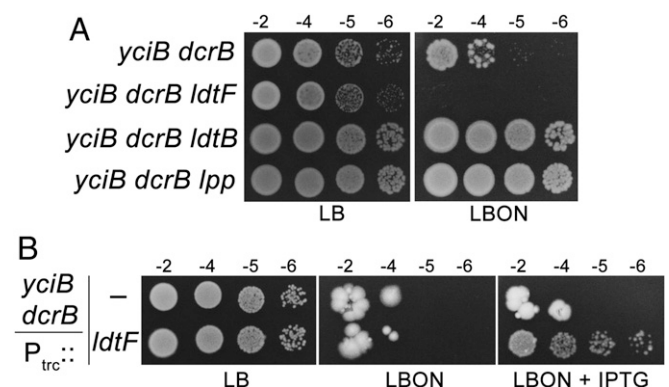
**Regulation of PG–Lpp Linkages.** Lpp is an abundant OM lipoprotein (5, 6, 10) with one-third of it covalently attached to PG, making almost 10% of the peptides linked to Lpp. However, it is not clear how *E. coli* maintains optimal levels of PG–Lpp linkages. The combined activities of LdtABC and LdtF may control the abundance of PG–Lpp linkages, or, alternatively, structural/conformational constraints of PG sacculi may limit the extent of PG–Lpp cross-link formation. These linkages are reported to be higher during conditions of stress, including the stationary phase of growth and certain mutant conditions (2, 16–18). LdtF-promoter expression is shown to be higher in the stationary phase (18); however, preliminary experiments done in our laboratory to examine the endogenous LdtF expression (using an LdtF–FLAG fusion) show that the protein levels fall during the stationary phase, suggesting a likely basis for the occurrence of a higher amount of PG–Lpp linkages in the stationary phase. It would be worthwhile to further examine how cells achieve a dynamic yet balanced level of PG–Lpp linkages to maintain the structural and functional integrity of the cell envelope.

**Role of PG–Lpp Linkages in PG Enlargement.** The absence of PG–Lpp linkages either by deleting Lpp or LdtB or increasing the copy number of LdtF partially rescued the growth defects of an *mepS* deletion mutant (Fig. 2 A and C); however, the suppression was not very robust (*SI Appendix, Figs. S14 and S2*). In addition, other phenotypes of *mepS*, such as sensitivity to β-lactam antibiotic, mecillinam, or its synthetic lethality with deletion of *mepM* (23),

were not suppressed by deletion of Lpp or LdtB, suggesting that the absence of Lpp linkages may not significantly affect the process of PG enlargement. The lack of OM–PG tethering may perhaps alter the mechanical properties of the cell envelope and increase the flexibility of the PG sacculus, consequently resulting in a marginal growth advantage to *mepS* mutants in low osmolar conditions such as NA.

### Materials and Methods

**Media, Bacterial Strains, and Plasmids.** LB has 0.5% yeast extract, 1% tryptone, and 1% NaCl (36). LBON is LB without NaCl. NA has 0.5% peptone and 0.3% beef extract. Antibiotics were used at the following concentrations: ampicillin, 50 μg/mL; chloramphenicol, 30 μg/mL; and kanamycin, 50 μg/mL.



**Fig. 6.** LdtF may also cleave Lpp-mediated IM–PG linkages. (A) The *yciB dcrB* mutant and its *ldtF*, *ldtB*, or *lpp* deletion derivatives were subjected to viability assays on LBON at 30 °C. (B) Indicated strains were grown with or without IPTG (0.1 mM) on LBON at 37 °C.

**Molecular and Genetic Techniques.** Recombinant DNA and plasmid constructions were performed as per the standard methods. MG1655 genomic DNA was used as a template, Phusion high-fidelity DNA polymerase (New England Biolabs) was used for PCR amplifications, and the plasmid clones were confirmed by sequence analysis. P1 phage-mediated transductions and transformations were performed using standard methods (36). All strains are derivatives of MG1655 (Coli Genetic Stock Centre) unless otherwise indicated. Deletion mutations are from the Keio mutant collection (37).

**Determination of PG–Lpp Linkages in the PG Sacculi.** Lpp-bound PG sacculi were isolated from cultures of WT and  $\Delta$ *ldtF* mutant, treated with mutanolysin, and the soluble fraction was run on 15% SDS-PAGE followed by Western blotting using anti-Lpp antibody.

**Determination of Enzymatic Activity.** To examine the activity of LdtF, soluble muropeptides were incubated with either buffer or LdtF (4  $\mu$ M) at 30 °C for 16 h. Samples were heat inactivated, reduced with sodium borohydride, and separated by RP-HPLC. Lpp cleavage activity was examined by incubating

purified LdtF (4  $\mu$ M) with the Lpp-bound PG sacculi for 16 h in 25 mM Tris-HCl (pH 8.0) at 30 °C, followed by electrophoresis of the soluble fraction on SDS-PAGE and Western blotting with anti-Lpp antibody.

**Data Availability.** All study data are included in the article and/or supporting information.

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