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STATE-OF-THE-ART REVIEW

LD-transpeptidases: the great unknown among the peptidoglycan cross-linkers

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The peptidoglycan (PG) cell wall is an essential polymer for the shape and viability of bacteria. Its protective role is in great part provided by its mesh-like character. Therefore, PG-cross-linking enzymes like the penicillin-binding proteins (PBPs) are among the best targets for antibiotics. However, while PBPs have been in the spotlight for more than 50 years, another class of PG-cross-linking enzymes called LD-transpeptidases (LDTs) seemed to contribute less to PG synthesis and, thus, has kept an aura of mystery. In the last years, a number of studies have associated LDTs with cell wall adaptation to stress including β-lactam antibiotics, outer membrane stability, and toxin delivery, which has shed light onto the biological meaning of these proteins. Furthermore, as some species display a great abundance of LD-cross-links in their cell wall, it has been hypothesized that LDTs could also be the main synthetic PG-transpeptidases in some bacteria. In this review, we introduce these enzymes and their role in PG biosynthesis and we highlight the most recent advances in understanding their biological role in diverse species.

Introduction

Most bacteria are surrounded by a cell wall, which provides mechanical strength to counteract the intracellular osmotic pressure, defines the cell shape, and also acts as a physical barrier against environmental insults [1,2]. A major component of the cell wall is the peptidoglycan (PG), also known as the murein sacculus, a net-like heteropolymer made of glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by β-1,4-glycosidic bonds and cross-linked by short peptides [3]. These disaccharide-peptide units that compose the PG are termed muropeptides. In Gram-negative bacteria, the cell wall is mainly single-layered and lies in the periplasmic space between the inner membrane (IM) and outer membrane (OM). Conversely, there is no OM in Gram-positive bacteria and a thick multilayered PG encompasses the cytoplasmic membrane (Fig. 1A).

As the PG is essential for bacteria and it is absent from eukaryotic cells, its synthesis is an excellent target of many antibiotics, such as the extensively used β-lactams and glycopeptides, which inhibit peptide cross-linking [4,5]. In *E. coli*, the majority of peptide cross-links are of 4-3 type [6,7], or also known as DD-type because they are catalyzed by the DD-transpeptidase activity of certain penicillin-binding proteins (PBPs) such as the bifunctional high molecular weight class A PBPs (PBP1a, PBP1b, and PBP1c) or the

Abbreviations
IM, inner membrane; LDT, LD-transpeptidase; Lpp, lipoprotein; LPS, lipopolysaccharide; *m*DAP, *meso*-diaminopimelic acid; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NCDAA, non-canonical D-amino acid; OM, outer membrane; PBP, penicillin-binding protein; PG, peptidoglycan.

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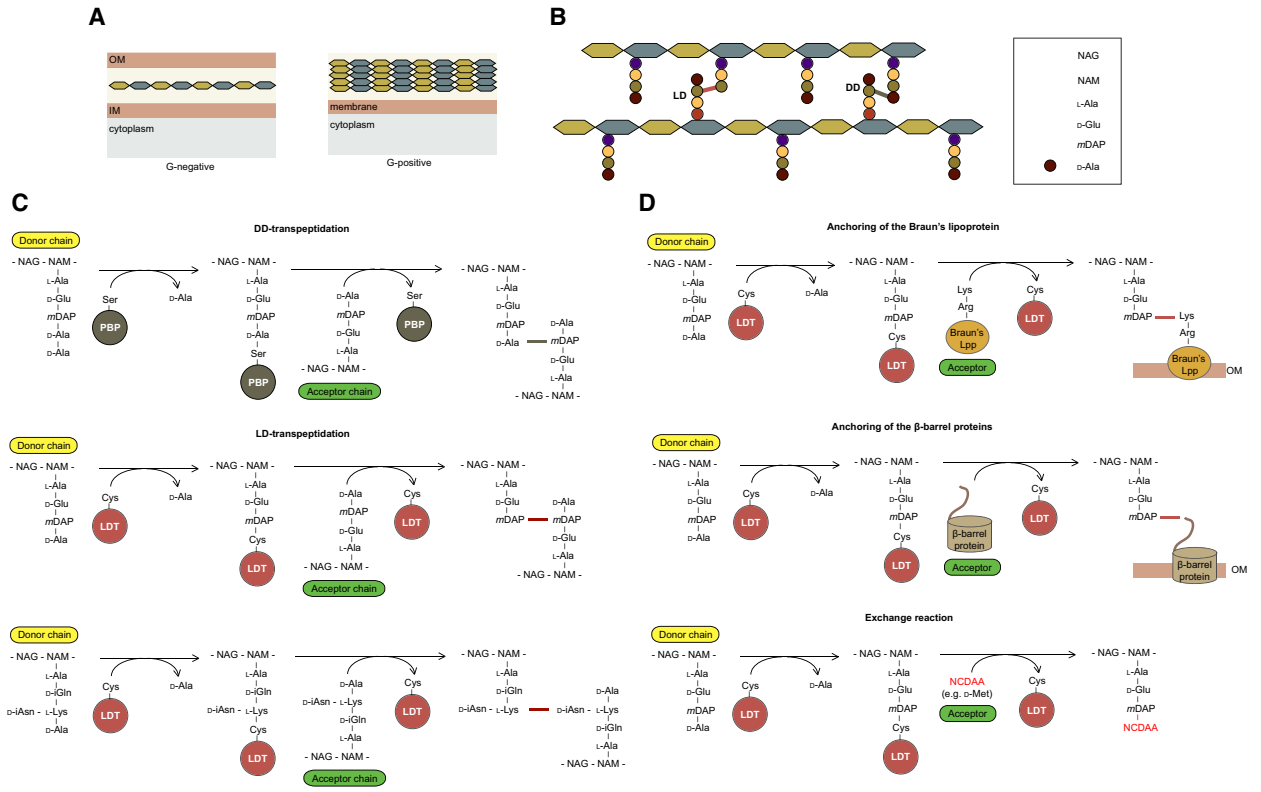

Fig. 1. Cell wall and PG structure. (A) Gram-negative bacteria possess mainly a single-layered PG confined in the periplasmic space between the IM and the OM. Gram-positive bacteria lack OM and produce a thick multilayered PG. (B) The canonical PG structure of Gram-negative bacteria is made of glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by β-1,4-glycosidic bonds and cross-linked by short peptides. Peptide cross-links of the DD type (aka 4-3 type) are catalyzed by the DD-transpeptidase activity of certain PBPs such as the bifunctional high molecular weight class A PBPs (PBP1a, PBP1b, and PBP1c) or the monofunctional class B PBPs (PBP2 and PBP3). The production of LD-cross-links (or 3-3 cross-links) depends on another type of enzyme, LDTs. (C) During DD-transpeptidation, PBP's catalytic nucleophilic Ser residue attacks the amide linkage between D-Ala^4 and D-Ala^5 in the donor pentapeptide, which releases D-Ala^5 and forms a covalent ester-based intermediate between the active Ser of the PBP and the tetrapeptide chain. Next, *m*DAP^3 in the peptide stem of the acceptor muropeptide nucleophilically attacks the ester linkage, forming a new amide bond and releasing the Ser residue of the PBP. During LD-transpeptidation, Ldt's catalytic Cys carries out a nucleophilic attack on *m*DAP^3 of the donor tetrapeptide, which releases D-Ala^4 and forms an acyl-enzyme intermediate. This acyl-enzyme intermediate is deacylated via nucleophilic attack of an amino group in the *m*DAP^3 side chain in the acceptor muropeptide, resulting in a *m*DAP^3-*m*DAP^3 bond and releasing the Cys residue of the LDT. In some organisms (e.g., *E. faecium*), LDTs catalyze the formation of cross-links through an interpeptide bridge. (D) In addition to the formation of LD-cross-links between muropeptides, LDTs are also involved in linking PG to Braun's Lpp and β-barrel proteins, as well as in the incorporation of NCDAAs into the PG.

monofunctional class B PBPs (PBP2 and PBP3) [8]. During DD-transpeptidation, the terminal D-Ala from a pentapeptide is cleaved and a new peptide bond (cross-link) is formed between the fourth D-Ala of this (donor) muropeptide and the *meso*-diaminopimelic acid (*m*DAP) in the third position of the neighboring, acceptor muropeptide. In *E. coli*, the function of bifunctional PBP1A and PBP1B requires interaction with their cognate OM-anchored lipoprotein LpoA and LpoB, respectively [9,10], which induces conformational changes that stimulate the activity of these PBPs [10–13].

While in *E. coli* like in most bacteria, the 4-3 type is the dominant PG cross-link, a smaller but significant proportion depends on another type of enzymes called LD-transpeptidases (LDTs; about 3%–15% in *E. coli* depending on the strain, growth condition, and phase) [6,7]. As the name of the enzyme suggests, LDTs catalyze the formation of a peptide bond between an L and a D-chiral center of 2 adjacent *m*DAP, so-called 3-3 or LD-type. Contrary to PBPs that use pentapeptides, LDTs use tetrapeptides (produced from pentapeptides by the DD-carboxypeptidase activity) as donor muropeptides and cleave the terminal (fourth)

D-Ala to cross-link the adjacent *m*DAP residues [6,7] (Fig. 1B,C).

LDTs do not share sequence homology with PBPs [14,15]; instead, they present a YkuD-like domain (PFAM 03744) that includes a cysteine as a catalytic nucleophilic residue instead of the catalytic serine in PBPs. The YkuD domain is an LDT catalytic domain that is named after the *Bacillus subtilis* YkuD protein, which is the first protein of its kind with a known crystal structure [16]. Moreover, contrary to PBPs, LDT enzymes are not efficiently inhibited by most β-lactams, although they are inactivated by carbapenems, penems, and to a lesser extent by cephalosporins through acylation of their catalytic site [17–20]. Although the first LD-transpeptidase was reported from an ampicillin-resistant strain of *Enterococcus faecium* [14], LDT orthologues have been found both in Gram-negative and Gram-positive bacteria [16,21–23]. The number of putative LDT proteins varies between species. For instance, *Neisseria meningitidis, Campylobacter jejuni*, and *Helicobacter pylori* encode only one putative LDT while homologs in *Agrobacterium tumefaciens* have 14 putative LDTs, 18 in *Mesorhizobium loti*, and 21 in *Bradyrhizobium japonicum* [24]. *E. coli* has six LDTs: LdtA-F. LtdD and LtdE form 3-3 cross-links, while LdtA, LdtB, and LdtC have a role in stabilizing the cell envelope by cross-linking the OM-anchored Braun's lipoprotein (Lpp) to the PG (Fig. 1D) [22,25]. LdtF is an amidase, which cleaves Braun's Lpp from PG [26,27], and is known to be involved in biofilm formation in pathogenic *E. coli* and seems to have a higher affinity for meropenem [28,29]. In addition to these activities, LDTs are also known to be able to catalyze exchange reactions where the usual terminal D-Ala in tetrapeptides can be replaced by another non-canonical D-amino acid (NCDAA) [25,30,31] (Fig. 1D). Although LDTs are nonessential in *E. coli*, they are involved in resistance to broad-spectrum β-lactams and in strengthening the bacterial cell envelope in response to defects in the OM [30,32,33]. However, in some other bacteria LD is the predominant cross-linking type and has more evident phenotypical consequences. This is the case of polarly growing bacteria such as mycobacteria (ca. 60% of LD cross-linkage) and *Agrobacterium tumefaciens* (> 50% of LD cross-linkage) [34–37], where LDTs seem to play an important role in the growth and shape maintenance [24,38]. Therefore, LDTs from diverse bacteria became the subject of structural studies and computational modeling, quantum mechanics, and molecular mechanics studies to dissect their substrate recognition and inhibition by drugs [16,39,48,40–47].

This review describes general and species-specific functions enabled by LD cross-links such as those related to adaptation to β-lactams stress. Additionally, we discuss how the capacity of the LDTs to incorporate D-amino acids has inspired a transformative tool to study PG growth and segregation in very diverse bacterial species.

β-lactam resistance mediated by LDTs

Mechanisms of LDT-mediated resistance vary in bacteria

LDT enzymes are not efficiently inhibited by most β-lactams and, thus, might support resistance to β-lactam antibiotics. This is the case of an *E. faecium* strain in which the essentiality of the PBPs can be bypassed by the ampicillin-insensitive Ldt_{fm} [49]. This resistance also requires a metallo-D,D-carboxypeptidase (DdcY), which by cleaving the terminal D-Ala in a UDP-MurNAc pentapeptide produces the precursor tetrapeptide substrate for the LDT activity [50]. Interestingly, this DD-carboxypeptidase is silent in the parental strain and is encoded by a cryptic locus, *ddc*, together with the two-component system DdcSR. The two-component system response regulator DdcR consists of an N-terminal effector domain and a C-terminal DNA-binding domain that belongs to the OmpR-PhoB subclass [51]. The sensor DdcS contains two clusters of hydrophobic amino acids that might correspond to trans-membrane segments delineating a periplasmic region of 26 residues at its N-terminal and a kinase domain at the C-terminal. Activation of the *ddc* locus in *E. faecium* strain results from a mutation in the sensor kinase gene *ddcS*, which impairs the DdcS phosphatase activity and leads to constitutive expression of the DD-carboxypeptidase [50]. Additionally, full bypass of PBPs by Ldt_{fm} also requires increased Ser/Thr protein phosphorylation (resulting from the impaired activity of phosphoprotein phosphatase StpA) [52]. Both DdcY production and the mutational alteration in the gene encoding StpA necessary and sufficient for high-level ampicillin resistance, since separately they provide only a moderate resistance level. However, StpA and its cognate serine/threonine protein kinase Stk do not control the expression of the *ddc* locus or ldt_{fm} and the targets of the kinase relevant in β-lactam tolerance have not been identified yet [52]. It is suggested that the number of Ser/Thr phosphoproteins is on the order of 100–200 in *E. faecium* [52]; thus, the role of protein phosphorylation in PG synthesis and ampicillin resistance still needs to be determined.

In *E. coli*, elevated levels of LdtD (formerly YcbB) and the alarmones guanosine tetraphosphate and

guanosine pentaphosphate (collectively referred to as (p)ppGpp) allow growth in otherwise lethal concentrations of ampicillin [32]. In this condition, LdtD also requires the DD-carboxypeptidase DacA (PBP5) to provide the tetrapeptide donor substrate and the glycosyltransferase activity of PBP1b (Fig. 2). These three proteins likely form a transient complex to rescue PG synthesis in the presence of β-lactam antibiotics [41]. Interestingly, copper inhibits LDTs by binding to the catalytic cysteine and prevents LDT-mediated β-lactam resistance in *E. coli* and *E. faecium* [33].

Structural studies on LDTs

Although LDTs are resistant to most β-lactams, (carba) penem antibiotics can inhibit their activity [32,53,54]. The molecular mechanisms responsible for this inhibition has become the subject of many structural studies, and as of today, there are 14 structures for unique proteins and multiple structures for proteins in complex with (carba)penems [16,40,41,43,53,55–59]. Depending on the species compared, the overall architecture of LDTs can be similar or vary in the domains present (Fig. 3) [40,41,43].

Recently, Caveney et al. reported the structure of *E. coli* LdtD with meropenem acylating the catalytic cysteine nucleophile (Cys528) [41]. The structure shows a monomeric form of the enzyme with the canonical catalytic LDT domain with the conserved active site motif ($HX_{15–18}$[S/T]XGCh[R/N], where 'X' represents any residue, and 'h' is any hydrophobic residue) [60], with the notable additions of a substrate capping subdomain, a PG-binding domain, and a large scaffold domain in the N-terminal region, which potentially plays a role in protein–protein interactions [41]. Despite certain architectural differences in the enzyme compared with other structurally characterized LDTs, the proposed catalytic mechanism for LdtD does not differ from what has been described for other LDTs before [58]. The conserved Cys528 carries out a nucleophilic attack on the third residue, *m*DAP, of the donor tetrapeptide, which results in the release of the terminal D-Ala. This acyl-enzyme intermediate is then deacylated via a nucleophilic attack of an amino group in the *m*DAP side chain in the acceptor. Comparison between the complexes of LdtD-meropenem and PBP5-meropenem [41] reveals that there is a distinct way of binding and stabilization of meropenem between the two enzymes.

Crystallographic studies of *Salmonella enterica* serovar Typhi and *Citrobacter rodentium* acylated LdtD homologs with ertapenem, the carbapenem antibiotic chemically related to meropenem, show that the overall enzyme structure and dimensions are similar to the one from *E. coli* [40].

It is noteworthy that LDTs of the same bacterium might vary in their size and domains. For instance, *Mycobacterium tuberculosis* five LDTs (Ldt_{Mt1-5}) have 30%–50% sequence identity, and four of them are structurally well characterized [53,55–58]. Ldt_{Mt2} and Ldt_{Mt5} are significantly longer than Ldt_{Mt1} and Ldt_{Mt3} and possess an extra domain: the bacterial immunoglobulin-like domain BIgA [55]. The presence of this additional domain might play a role of a pedestal to approximate the catalytic site of membrane-attached LDTs to their substrates located in different PG layers [61]; on the contrary, different LDTs in *M. tuberculosis* could also co-localize in different parts of the cell wall and play specific functions to the PG maintenance there.

Such structural and computational studies on the enzymes and their complexes with antibiotics might provide insights into the inhibition mechanisms and lead to the development of novel inhibitors to combat the β-lactam resistance crisis.

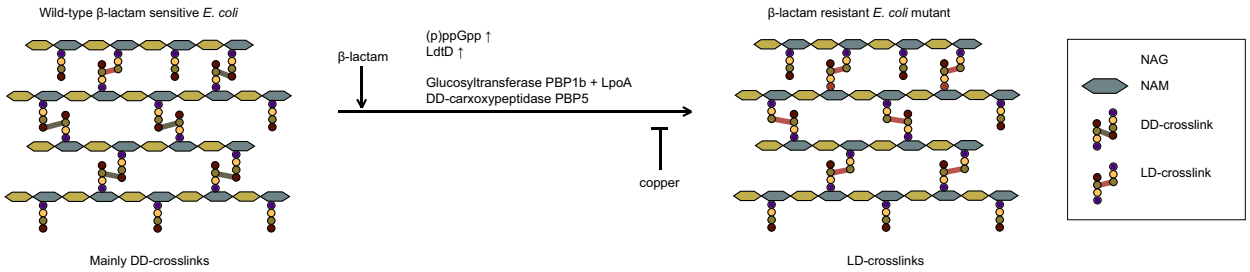

Fig. 2. LDT provides resistance to β-lactam antibiotics in *E. coli*. Elevated levels of LdtD and the alarmones guanosine tetraphosphate and guanosine pentaphosphate ((p)ppGpp) together with the glycosyltransferase activity of PBP1b (and LpoB) and DD-carboxypeptidase DacA (PBP5) rescue PG synthesis when DD-transpeptidation is inhibited by β-lactams. The addition of copper inhibits the LDTs and so prevents β-lactam resistance.

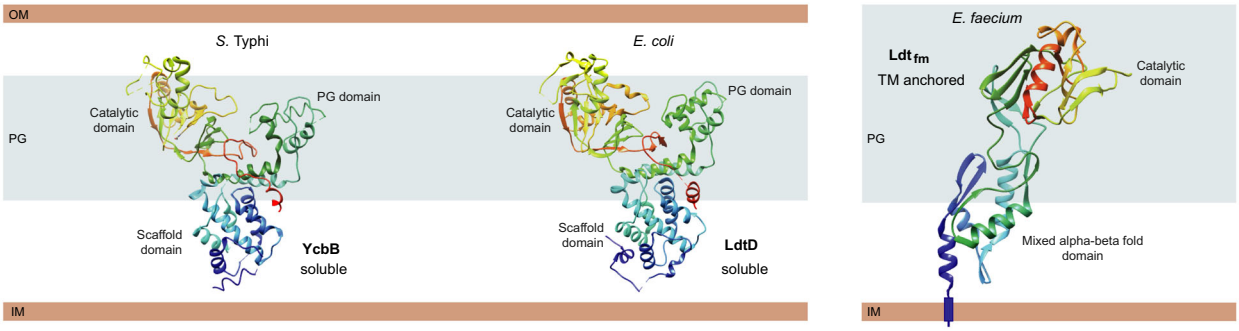

Fig. 3. A comparison of structurally characterized LDTs. The overall architecture of LDT proteins in different bacteria might show a high degree of similarity or be quite distinct as seen from this example of *S.* Typhi YcbB (7KGN [40]), *E. coli* LdtD (6NTW [41]), and *E. faecium* LDT_{fm} (1ZAT [43]). *E. coli* LdtD and LdtD homolog in *S.* Typhi, YcbB, both feature a three-domain format with the catalytic domain with substrate capping subdomain, PG, and scaffolding domains. *E. faecium* Ldt_{fm} consists of two domains: an N-terminal domain with a mixed alpha-beta fold, and the C-terminal catalytic domain. Protein structures are colored in a rainbow palette from N (blue) to C terminus (red). Protein molecular graphics were performed with UCSF Chimera [102].

LD-cross-links strengthen the PG layer under stress conditions

LDTs reinforce PG during OM assembly defect

Defects in the OM may lead to local mechanical stress on the PG. In *E. coli*, LDT activity is required to avoid lysis when lipopolysaccharide (LPS) transport to the OM is compromised [30]. LdtD, the major PG synthase PBP1B, its activator LpoB, and the DD-carboxypeptidase PBP6a seem to cooperate to strengthen the PG layer and to rebalance the mechanical load between PG and OM [30].

The major role of LdtD in PG remodeling during cell envelope stress is in concordance with its upregulation by the Cpx-mediated stress response [62,63]. In *E. coli*, Cpx is one of the primary envelope stress response systems (ESRS), which span the envelope, sense perturbations, and induce repair or preventive action by changing the expression of appropriate genes [64–66]. The core machinery of the Cpx pathway is formed by a classical two-component system: envelope stress triggers a phosphotransfer between the sensor histidine kinase CpxA at the IM and the cytoplasmic response regulator CpxR, which regulates the transcription of diverse genes [67,68]. In contrast to LdtD, which is activated under envelope stress conditions, another LDT forming 3-3 cross-links, LdtE, is regulated by RpoS, the alternative sigma factor for stationary-phase gene expression [69], and is maximally expressed in stationary-phase cells [30]. Interestingly, LdtF (renamed from YafK), an amidase that cleaves Braun's Lpp from PG [26,27], is also RpoS regulated and seems to stimulate the activity of both LdtD and LdtE [30]. In unstressed cells in which LdtD is poorly expressed, LdtE-LdtF couple forms most of the 3-3 cross-links and therefore LdtE and LdtF are housekeeping LDTs [30]. Deletion of *ldtE* and *ldtF* leads to activation of stress LDT, LdtD, and increased levels of 3-3 cross-links [30].

This functional connection between OM biogenesis and PG remodeling emphasizes the versatile mechanisms that bacteria employ to maintain cell envelope integrity under a variety of growth and stress conditions.

LDTs protect plant-associated bacteria from osmotic shock

Plant pathogens that reside in the phloem must continually adapt to osmotic pressure changes, since sucrose concentration in the phloem can fluctuate between 15 and 880 mM, depending on plant species, tissue, time of the day, and season [70,71]. Pagliai et al. [72] show that in the unculturable citrus pathogen *Candidatus* Liberibacter asiaticus the transcriptional activator LdtR mediates tolerance to osmotic stress through the predicted LD-transpeptidase *ldtP*. Due to *Ca.* L. asiaticus unculturability, several bacterial model strains were used to confirm these results. Disruption of *ldtR* and *ldtP* in the close phylogenetic relative *Sinorhizobium meliloti* revealed that the cells are shorter than wt and more sensitive to osmotic shock. Furthermore, the binding of LdtR to the *ldtP* promoter can be inhibited by certain small molecules such as phloretin, benzbromarone, and hexestrol [72]. Treatment of *Rhizobiaceae* family bacteria, such as *S. meliloti* and *Liberibacter crescens* (another *Ca.* L. asiaticus close relative) with these molecules induced cell shortening and increased sensitivity to osmotic challenge suggesting

that these small molecules can be used as a potential treatment of the citrus disease caused by *Ca*. L. asiaticus [72]. Interestingly, in addition to LdtP's PG remodeling activity under osmotic stress, this enzyme also displays esterase activity toward the short-chain carboxyl esters in solution as was demonstrated with model substrates *p*-nitrophenyl acetate and butyrate, and putatively is able to modify the lipid A moiety of the LPS likely by eliminating acyl chains with its esterase activity [73]. This moonlighting activity might be a consequence of the evolutionary genomic reduction to adapt to the intracellular lifestyle in the phloem and to avoid recognition by the plant immune system.

LD-cross-links might provide lysozyme resistance

Caulobacter crescentus has an unusual morphology and dimorphic life cycle [74]. During the sessile stage, this bacterium displays a polar stalk with an adhesive holdfast, which mediates surface attachment. Interestingly, the polar stalk is lysozyme resistant, and its PG composition differs from that of the main body [75,76]. While the PG of the main body is primarily DD-cross-linked, the PG of the stalk PG is enriched in LD-cross-links, mainly mediated by LdtD. Deletion of *ldtD* does not affect stalk elongation; however, it increases stalk sensitivity to lysozyme [75]. In fact, there is a certain correlation between the amount of LD-cross-linkage and sensitivity to lysozyme in other Gram-negative bacteria as well. For instance, *A. tumefaciens* has a PG rich in LD-cross-links and is also highly resistant to lysozyme degradation [75]. In *E. coli*, the lower susceptibility to lysozyme of stationary-phase cells correlates with an increase in LD-cross-linkage increases during the transition from log to stationary phase (3.5%–8.6%).[6,75] In *Corynebacterium glutamicum*, deletion of the σ^D-dependent LDT, lppS, leads to increased sensitivity to lysozyme [77]. A triple LDT mutant shows also increased susceptibility to lysozyme in *Mycobacterium smegmatis* [78].

The correlation between LD-transpeptidation and lysozyme resistance might not be very intuitive since lysozyme hydrolyzes the β- (1-4) glycosidic bond between NAM-NAG, whereas LD-cross-links occur between peptide stems. Computational modeling supports a more rigid and extended conformation of LD-cross-linked muropeptides, in part due to the presence of the fourth D-Ala and additional hydrogen bonds, which favors a more compact stem peptide folding in DD-cross-linked muropeptides [79]. These hallmarks imply that each kind of cross-link might have a different relative orientation and distance to the glycans [79], which in turn might affect the accessibility of the lysozyme to glycan strands. Altogether this means that the relative abundances of different PG cross-linking types might be regulated to adapt to different situations and, thus, standard laboratory conditions might often overlook the importance of a particular set of enzymes for the growth and fitness of bacteria.

Local LD-cross-links are involved in species-specific functions

LD-cross-linked PG establishes a stable intracellular niche

Bdellovibrio bacteriovorus is a micro-predator (approximately 1.0 × 0.3 μm) that feeds upon and proliferates inside other (larger) Gram-negative bacterial species [80]. *B. bacteriovorus* colonizes the periplasm of the prey by breaching the OM. At this stage, the prey cell becomes rounded forming a bdelloplast [81], and two out of 19 LDT genes in *B. bacteriovorus* are upregulated (genes *bd0886* and *bd1176*) [82]. The activity of these two LDTs induces modifications in the PG of the prey that improve its physical robustness, as it was demonstrated by challenging the resistance to osmotic stress of bdelloplasts produced by wild-type *B. bacteriovorus* compared with those of the Δ*bd0886*Δ*bd1176* mutant. In the fortified bdelloplast, *B. bacteriovorus* digests host resources, grows, and eventually divides [80] to finally burst out free to invade new prey and repeat the cycle.

LDT modifies PG at the poles for typhoid toxin translocation

Typhoid toxin is an essential virulence factor for the human pathogen *S*. Typhi and the cause of typhoid fever in humans [83]. Produced only within mammalian cells, translocation of this toxin across the PG requires a specialized polarly localized muramidase (TtsA) and PG editing at the bacterial poles by LDT YcbB (homolog of *E. coli* LdtD, which was formerly named YcbB) [84] (Fig. 4). TtsA carboxy-terminus domain is essential for polar localization and substrate recognition [85]. LDT-modified PG *per se* is not required for polar localization of TtsA. Translocation of the toxin positions it in close proximity to the OM from where it can be released through minor disruptions in the membrane caused by membrane-active compounds (such as antimicrobial peptides or bile salts) encountered by *S*. Typhi during infection [84].

In all, this could mean that in some bacteria LDTs might not be the part of main PG synthesis; nevertheless, they are important players during certain developmental stages or crucial for specific functions.

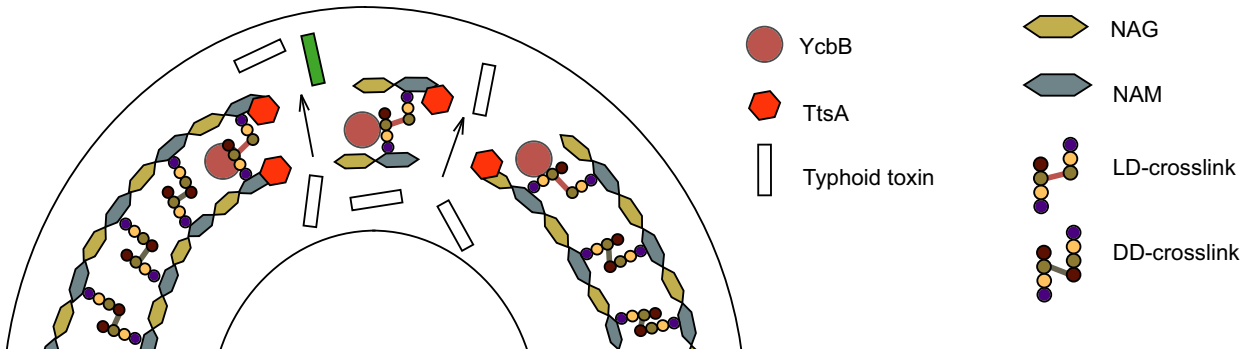

Fig. 4. Model of typhoid toxin translocation across the PG. Translocation of assembled typhoid toxin from the *cis* to the *trans* side of the PG occurs at the poles and relies on the muramidase TtsA. TtsA activity requires PG editing at the poles by LDT YcbB. Scheme adapted from [84].

LDTs cross-link the OM and PG

Three out of the six LDTs in *E. coli*, ErfK, YbiS, and YcfS, renamed LdtA, LdtB, and LdtC, respectively, covalently attach the abundant OM-anchored Braun's Lpp to *m*DAP residues in PG [25,86]. In *E. coli* and closely related bacteria, Lpp is required to maintain the periplasmic width between OM and IM [87]. Disruption of this spacing interferes with the proper assembly of periplasm-spanning structures and with signal transduction from the environment to the cell [88,89]. Recently, it was shown that in some environmental pathogens (*Coxiella burnetii*, *A. tumefaciens*, *Legionella pneumophila*, *Brucella abortus*) that lack Lpp orthologues, multiple OM β-barrel proteins are covalently attached to the PG [90,91]. In the intracellular pathogen, *C. burnetii* one (Cbu0318) out of the ten predicted LDTs is involved in the attachment of β-barrel proteins BbpA and BbpB to the PG, which stabilizes the OM of this bacterium during the stationary phase [90]. Also, in the intracellular pathogen *B. abortus*, the Ldt4 (BAB1_0589) is the main LDT required to interlink the OM and the PG [91]. It is hypothesized that bacteria that alternate between host(s) and environment might require multiple OM tethers since they need to survive extreme changes in pH, temperature, and osmolarity [92].

LDTs incorporate NCDAAs into PG

Incorporation of NCDAAs by LDTs changes cell wall properties and regulation

In addition to catalyzing LD-cross-linkage formation, LDTs are also able to incorporate a variety of NCDAAs in the place of D-Ala in the fourth position in the muropeptides [31]. Bacteria from diverse environments produce NCDAAs through the activity of broad-spectrum racemases [93]. In *Vibrio cholerae*, the etiological agent of the diarrheal disease cholera, LDT-mediated incorporation of these D-amino acids into the PG controls PG strength and amount during the stationary phase [31]. Furthermore, *V. cholerae* releases NCDAA-modified tetrapeptides during PG turn-over, which are later reincorporated by the PG recycling pathway. However, NCDAA-tetrapeptides are poor substrates for the PG recycling enzyme LD-carboxypeptidase and their cytosolic accumulation also downregulates negatively the PG synthesis [94], in a coordinated manner with the periplasmic PG editing by LDTs. Interestingly, the incorporation of NCDAAs by LDTs is more widespread than NCDAA production [31]. Therefore, potentially NCDAAs and NCDAA-edited muropeptides might be used to mediate bacterial communications (competition or cooperation) through their incorporation by LDTs [94,95].

D-amino acid-based probes incorporated by LDTs allow PG visualization

The activity of LDTs (as well as PBPs) to catalyze periplasmic exchange reactions to achieve the incorporation of NCDAAs into the PG inspired the use of synthetic D-amino acid-based probes to visualize PG assembly and metabolism in a precise spatial and temporal manner in nearly all PG-containing bacteria [96–99]. The incorporation of these probes into the PG depends on each specific organism. For instance, in *E. coli* single D-amino acid-based fluorescent probes or FDAA (e.g., EDA, HADA) are incorporated

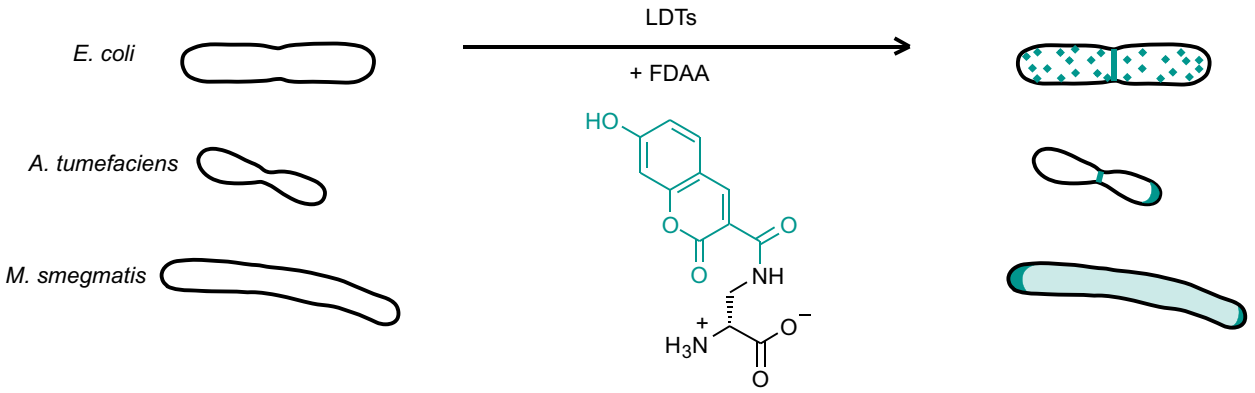

Fig. 5. Fluorescent D-amino acid (FDAA) labeling of bacteria through LDTs incorporation of the molecule into PG. FDAA labeling pattern reveals the mode of PG assembly and metabolism in the specific bacterium: *E. coli* makes use of dispersed growth, which is seen as punctate patterns on the lateral walls of elongating cells, *A. tumefaciens* grows from the new cell pole (the pole formed at the last cell division), in *M. smegmatis* new PG is unequally inserted at both poles (more at the old pole).

mainly by the activity of LDTs into the muropeptides fourth position, while in vegetative *B. subtilis* cells this process depends on the activity of PBPs [100].

The incorporation of FDAA into the PG provides valuable information about how the cell wall grows. As in *E. coli*, in *A. tumefaciens* HADA is also mainly incorporated into the fourth position of the peptide moieties, but they show completely different labeling patterns [96]. In *E. coli* short labeling with HADA shows fluorescence localization at the septal plane of predivisional cells and in punctate patterns on the lateral walls of elongating cells [96]. However, in *A. tumefaciens* the signal predominantly localizes at the new pole and the site of cell division (Fig. 4) [96]. It has been shown that *A. tumefaciens*' LDT Atu0845 shares this same localization pattern [24], thereby suggesting that LDTs play a role in polar growth in this bacterium.

In *Mycobacterium smegmatis*, FDAAs are incorporated asymmetrically by LDTs [38]. A short pulse of FDAAs produces fluorescence signal at both poles (more at the old pole), where new PG is inserted in mycobacteria [101], and a gradient along the sidewalls, which extends from the old pole and fades around the mid-cell as it reaches the new pole (the pole that was formed at the last cell division) [38] (Fig. 5). Deletion of all 6 LDTs in *M. smegmatis* results in nearly abolished FDAAs labeling and loss of rod shape [38]. These cells produce spherical blebs, which likely correspond to sites of old and nonuniformly distributed cell wall as a result of the asymmetric polar growth and division. It is hypothesized that these blebs emerge in the mutant because LDTs would normally reinforce aging PG to maintain rod shape [38]. A large number of publications have supported the use of FDAA as an excellent method to track spatiotemporal cell wall growth and segregation in many diverse bacteria. Given that PG is a common target of antibiotics, this visual, in vivo-compatible tool, might aid in the development of new treatment strategies.

Future perspectives

The roles of LDTs in different bacteria under standard laboratory conditions vary from dispensable to essential, which places them in the position of accessory proteins or proteins that participate in the main PG synthesis. However, the role of LDTs seems to gain importance in bacterial physiology under stress conditions. This is not surprising, since many bacteria face extreme fluctuations in their natural habitats (e.g., temperature, pH, salinity, availability of nutrients, and small signaling molecules), often associated with free-living to host(s)-associated transitions. Therefore, analysis under non-physiological conditions such as optimal temperature and nutrient-rich media must be complemented with more challenging, suboptimal conditions to better understand the biological role of LDTs. Moreover, many bacteria possess multiple copies of LDTs (even more than 20) [24], which might even represent an additional level of functional specialization between enzymes that a priori share the same activity. Could it be that in these bacteria different LDTs belong to different PG synthetic protein complexes and regulatory networks? Do different LDTs have different preferred substrates? How are LDTs regulated in the cells? What has been the natural selective pressure that has driven the emergence of LDTs? There are many yet unanswered questions in this field.

Thus, coming research efforts will be heading to figure out the unique and conserved properties of LDTs in bacteria, and the role that each LDT plays in a specific organism and its functional significance.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Both authors made a substantial, direct and intellectual contribution to the work and approved it for publication.

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