Peptidoglycan Remodeling Enables *Escherichia coli* To Survive Severe Outer Membrane Assembly Defect

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ABSTRACT Gram-negative bacteria have a tripartite cell envelope with the cytoplasmic membrane (CM), a stress-bearing peptidoglycan (PG) layer, and the asymmetric outer membrane (OM) containing lipopolysaccharide (LPS) in the outer leaflet. Cells must tightly coordinate the growth of their complex envelope to maintain cellular integrity and OM permeability barrier function. The biogenesis of PG and LPS relies on specialized macromolecular complexes that span the entire envelope. In this work, we show that *Escherichia coli* cells are capable of avoiding lysis when the transport of LPS to the OM is compromised, by utilizing LD-transpeptidases (LDTs) to generate 3-3 cross-links in the PG. This PG remodeling program relies mainly on the activities of the stress response LDT, LdtD, together with the major PG synthase PBP1B, its cognate activator LpoB, and the carboxypeptidase PBP6a. Our data support a model according to which these proteins cooperate to strengthen the PG in response to defective OM synthesis.

IMPORTANCE In Gram-negative bacteria, the outer membrane protects the cell against many toxic molecules, and the peptidoglycan layer provides protection against osmotic challenges, allowing bacterial cells to survive in changing environments. Maintaining cell envelope integrity is therefore a question of life or death for a bacterial cell. Here we show that *Escherichia coli* cells are capable of avoiding lysis when the transport of LPS to the OM is compromised, by utilizing LD-transpeptidases (LDTs) to generate 3-3 cross-links in the peptidoglycan layer when the integrity of the outer membrane is compromised, and this response is required to avoid cell lysis. This peptidoglycan remodeling program is a strategy to increase the overall robustness of the bacterial cell envelope in response to defects in the outer membrane.

KEYWORDS *Escherichia coli*, cell envelope, lipopolysaccharide, peptidoglycan, stress response

The integrity of a diderm (Gram-negative) bacterial cell is maintained by a complex cell envelope composed of the cytoplasmic membrane (CM), the periplasm with a thin peptidoglycan (PG) sacculus, and the outer membrane (OM) (1, 2). The asymmetrical OM contains in the outer leaflet lipopolysaccharide (LPS) (3), which makes the cell envelope impermeable to many toxic compounds and antibiotics (4).

LPS is assembled at the outer leaflet of the CM (5–7) and then transported across the periplasm to reach its final destination at the outermost surface of the cell (8, 9). In *Escherichia coli*, LPS transport is facilitated by seven essential proteins, LptA to LptG (10–15) which form a transenvelope protein bridge through the periplasm and its PG sacculus (11, 16–18). This organization allows the coupling of ATP hydrolysis with LPS
movement across the periplasm up to the cell surface, as proposed in the so-called PEZ model (19). Depletion of any of the Lpt components results in block of LPS transport and its accumulation at the periplasmic leaflet of the cytoplasmic membrane (CM) (12, 14).

LPS export to the OM is among the cell’s main transport processes. Considering a generation time of 20 min for fast growing *E. coli*, LPS transport must occur at a rate of more than $10^3$ molecules per second to ensure complete coverage of the cell surface during growth (20). Moreover, the supply of LPS must be optimally coupled to the synthesis and assembly of other cell envelope components, such as PG, to prevent loss of OM integrity due to LPS depletion or detrimental effects by excessive LPS production. How LPS synthesis and export is regulated remains largely unknown.

LPS is exported through the periplasmic PG sacculus that has a net-like structure composed of glycan strands connected by short, cross-linked peptides (2, 21). PBP1A and PBP1B are major and semiredundant PG synthases that polymerize glycan strands by their glycosyltransferase (GTase) activity and cross-link stem peptides by DD-transpeptidase (DD-TPase) activity, forming the abundant 4-3 cross-links in PG (see Fig. S1A in the supplemental material) (22–24). The CM-anchored PBPs require activation by their cognate, OM-anchored lipoprotein (LpoA and LpoB, respectively) (25–27). LpoA and LpoB span the periplasm to activate their cognate PBP (28–30), presumably responding to the size of pores in the PG layer to couple PG growth with cell growth (21).

DD-carboxypeptidases (DD-CPase) such as PBP5, PBP6a, and PBP6b trim the penta-peptides present in new PG to tetrapeptides (31–33). PBP5 is the major DD-CPase in the cell; its absence causes aberrant cell morphology in strains lacking other PBPs (33, 34). PBP6b contributes substantially to PG remodeling and cell shape maintenance in cells growing at acidic pH (35).

In *E. coli*, the majority (90% to 98%) of cross-links in PG are of the 4-3 (or DD) type (between D-Ala and meso-diaminopimelic acid [meso-Dap]) (36). However, there are 2 to 10% of the 3-3 (or LD) type of cross-links between two meso-Dap residues of adjacent stem peptides (Fig. S1A), and these increase to up to 16% in stationary-phase cells (36, 37). 3-3 cross-links are produced by LD-transpeptidases (LDTs) of the *YkuD* family of proteins (PF03734), which are structurally unrelated to PBPs. LDTs use tetrapeptide donors in the TPase reaction and are insensitive to most β-lactams (Fig. S1A) (38).

*E. coli* has five LDTs with two distinct functions. LdtD (formerly YcbB) and LdtE (YnhG) form 3-3 cross-links, whereas LdtA (ErfK), LdtB (YbiS), and LdtC (YcfS) attach the abundant OM-anchored Lpp (Braun’s lipoprotein) to meso-Dap residues in PG, providing a tight connection between the PG and OM. Notably, *E. coli* mutants with multiple or all *ldt* genes deleted exhibit only minor phenotypes, suggesting that these functions are dispensable during growth under laboratory conditions (39–41).

Certain strains of *Enterococcus faecium* can grow in the presence of β-lactam antibiotics using a β-lactam-insensitive LDT, Ldt_m, to produce 3-3 cross-links instead of the β-lactam-sensitive PBP TPases (42–44). More recently, a DD-TPase-independent and LDT-dependent mutant strain of *E. coli* has been selected by its ability to grow at a high and otherwise lethal concentration of ampicillin, at which it produces exclusively 3-3 cross-links in its PG (45). This strain has an elevated level of the alarmone (pppGpp and needs LdtD, the DD-CPase PBP5, and the GTase domain of PBP1B together with its regulator, LpoB, to bypass PBPs and achieve broad-spectrum β-lactam resistance (45). However, *E. coli* strains do not readily acquire this mechanism of resistance, and it is possible that the 3-3 cross-linking activities of LdtD and LdtE have another, yet undiscovered function in *E. coli*.

In this work, we show that *E. coli* cells defective in the LPS export pathway require LDTs that produce an increased level of 3-3 cross-links in the PG to avoid cell lysis. Our data suggest that LdtD is specifically expressed in response to OM damage and participates in a PG remodeling program activated in response to the block of LPS transport. Notably, PG remodeling also involves the GTase activity of PBP1B and the DD-CPase of previously unknown function, PBP6a. We propose a model whereby
PBP1B, LdtD, and PBP6a cooperate in a dedicated PG machine which is needed when LPS transport is compromised.

RESULTS

Defective LPS export induces the formation of 3-3 cross-links in PG. We previously observed that several PG-synthesizing or PG-modifying enzymes are upregulated upon depletion of the essential LptC component of the LPS export machinery (46), prompting us to analyze the composition of PG isolated from cells with compromised LPS transport.

For this purpose, we cultured an araBiplptC conditional strain, in which lptC expression is under the control of the arabinose-inducible araBp promoter. As previously reported (13), LptC-depleted cells formed short chains and arrested growth (Fig. 1A and B). The sacculi purified from these cells showed a four- to sixfold increase in the relative amount of 3-3 cross-links between two meso-Dap residues compared to sacculi from cells grown in the presence of arabinose (Fig. 1E and Table 1; see also Table S3 in the supplemental material). 3-3 cross-links increased early in LptC-depleted cells, indicating a rapid cellular response to the LPS transport arrest. We also observed only a moderate decrease in the canonical 4-3 (meso-Dap to D-Ala) cross-links in LptC-depleted cells (Fig. 1E and Table S3).

3-3 cross-links are not essential under standard growth conditions. E. coli has five LDTs (LdtA to LdtE) (39–41). When inspecting the E. coli genome, we identified another hypothetical ldt gene, yafK. The predicted YafK shares 33% and 41% sequence identity to the catalytic YkuD (LDT) domain of LdtD and LdtE, respectively, but lacks a conserved arginine residue near the active site cysteine and might not be fully active (Fig. S1B). We included yafK (now termed ldtF) in our further studies on the roles of LDTs in the formation of 3-3 cross-links during defective LPS export.

To assess the roles of the LDTs in E. coli, we examined the growth phenotypes and levels of 3-3 cross-links in sacculi purified from all possible single and multiple deletion mutants. The deletion of ldtD, ldtE, and ldtF alone and in all possible combinations did not affect the growth of E. coli (Table 1 and Fig. S3A and B). Even the deletion of all six ldt genes did not result in any growth defect under standard laboratory conditions (data not shown). The muropeptide analysis revealed that only 3.0% of the PG muropeptides from strain BW25113 contained 3-3 cross-links (Table 1 and Table S3), consistent with earlier reports (36, 37). The ΔldtD ΔldtE mutant contained 2.2% muropeptides with 3-3 cross-links, and 3-3 cross-links were not detected in the PG from the ΔldtD ΔldtE ΔldtF triple mutant, suggesting that E. coli has no other enzyme for 3-3 cross-link formation in the absence of LdtD, LdtE, and LdtF. The ΔldtD ΔldtF double mutant did not produce detectable levels of 3-3 cross-links, suggesting that LdtE is either not active as an LD-TPase or it requires LdtD and/or LdtF for activity. In all other ldt defective strains, the level of 3-3 cross-links was comparable to that of the BW25113 wild-type strain, suggesting that one or more LDTs is active in these mutants (Table 1 and Table S3).

We next ectopically expressed ldtD, ldtE, and/or ldtF (Table S1) in an E. coli BW25113Δ6LDT background, which lacks ldtABCDEF (ldtA-F) (47), and analyzed the PG composition (Fig. 2). Expression of LdtD alone, but not expression of LdtE or LdtF, resulted in the presence of 3-3 cross-links in PG. Coexpression of LdtF with LdtD or LdtE increased the level of 3-3 cross-links (compared to LdtD or LdtE alone), suggesting that LdtF might not be an active LD-TPase but stimulates the other two enzymes. In line with this hypothesis, we found that 3-3 cross-links were not detected in a ΔldtA-E mutant that expressed ldtF as the sole ykuD homologue (Table S3).

LDTs prevent cell lysis upon defective OM assembly. Because the level of the 3-3 cross-links increased in LptC-depleted cells, we deleted every ldt gene alone and all possible combinations in the araBiplptC conditional mutant, and we examined the growth profile and level of 3-3 cross-links in the PG under permissive and nonpermissive conditions.
FIG 1 LDTs prevent cell lysis upon defective OM assembly. (A to D) Cells of the araBp{lptC} conditional strain (A and B) and the isogenic mutants with ldtD, ldtE, and ldtF deleted (C and D) were grown in the presence of 0.2% arabinose to an OD600

(Continued on next page)
 Upon shifting to the nonpermissive condition, LptC-depleted ldt mutant cultures (with the exception of ΔldtE ldtF mutant) decreased in optical density, and the cells lost viability as shown by their reduced ability to form colonies. Phase-contrast and fluorescence microscopy revealed bulges at variable positions on the cell surface, suggesting that the cellular integrity was compromised (Fig. 1C and F and Fig. S2 to S4). These effects were specific for the loss of LDTs forming 3-3 cross-links because the simultaneous removal of all Lpp attachment enzymes (ldtA-C deletion) did not result in lysis upon lptC depletion (Fig. S5A and B). In the lysis-prone lptC-depleted ΔldtD or ΔldtE mutants, the level of 3-3 cross-links was only slightly reduced compared to the araBplptC parental strain (Table 1 and Table S3). The lysis phenotype of araBplptC ΔldtD cells was rescued by ectopic expression of native LdtD, but not LdtDC528A in which the catalytic Cys residue is mutated to Ala (Fig. S5C), showing that the activity of LdtD is required to rescue cells from lysis upon OM defective assembly.

All strains with defective ldt genes lysed under nonpermissive conditions except the araBplptC ΔldtE ΔldtF mutant which arrested growth like the araBplptC parental strain (Fig. 1F and Fig. S4A and B). These cells displayed a high level (>8%) of 3-3 cross-links at all conditions (i.e., even without deletion of lptC) (Table 1). This suggests that LdtD is active and able to prevent lysis of these cells. In line with this finding, we indeed observed that ectopic expression of ldtD rescues all LptC-depleted single and multiple ldt mutant strains from lysis (Table 1 and Fig. SSD to H). Finally, the LptC-depleted ΔldtD mutant produced 3-3 cross-links and lysed at nonpermissive conditions (Table 1 and Fig. S3C and D), but in sharp contrast to the other strains, araBplptC ΔldtF cells showed morphological defects even when grown at permissive conditions (Fig. S3D), and no...
morphological defects were observed when ldtF was deleted in the lptC background (Fig. S3B), suggesting that the deletion of ldtF caused additional problems to cells with depleted LptC levels.

Lysis of LptC-depleted cells could be caused by the accumulation of LPS at the outer leaflet of the CM (11, 14). We therefore assessed whether the LptC depletion-induced lysis occurs in cells with blocked LPS synthesis due to inhibition of LpxC by LPC-058 (48). We observed lysis in BW25113 ΔldtD and araB-pLptC ΔldtD cells treated with LPC-058 but not in the corresponding parental strains (carrying a functional ldtD copy) treated with LPC-058 (Fig. 3A and B). These results suggest that lysis is not due to perturbation of the CM or periplasmic stress caused by depletion of a component of the Lpt machinery but is rather the consequence of lack of PG remodeling by LdtD.

The ldtD promoter is activated under envelope stress conditions. To assess how the ldt genes are regulated in the cell, we constructed transcriptional fusions of the promoter region of each ldt gene to lacZ, and the resulting plasmids with pldtD-lacZ, pldtE-lacZ, and pldtF-lacZ were introduced into strain BW25113, the conditional araB-pLptC mutant, and their derivatives with deletion of ldtD, ldtE, and ldtF alone and in all possible combinations. β-Galactosidase activity was measured in extracts from cells collected at different time points during growth.
The expression of *ldtE* and *ldtF* in the *lptC*<sup>−</sup> background was growth phase dependent. In the wild-type strain and the *araB*p*lptC* conditional mutant grown under permissive and nonpermissive conditions, *pldtE-lacZ* and *pldtF-lacZ* were maximally expressed in stationary-phase cells (Fig. 4B and C). Consistent with their expression pattern, *ldtE* and *ldtF* were both regulated by RpoS, the alternative sigma factor for stationary-phase gene expression (49), and both genes lost their growth phase-dependent promoter activation in a BW25113ΔrpoS mutant (Fig. 4D).

The *ldtD* promoter was not activated in the wild-type *lptC*<sup>−</sup> strain and in *ldt* derivatives with the exception of the Δ*ldtE* Δ*ldtF* mutant and was up to eightfold...
The *ldtD* promoter is activated under envelope stress conditions, and *ldtE* and *ldtF* are RpoS-regulated genes. Wild-type strain BW25113 (lptC<sup>+</sup>) and isogenic mutants with every *ldt* gene deleted alone and in all possible combinations were transformed with plasmids expressing *ldtD*-lacZ (A), *ldtE*-lacZ (B), or *ldtF*-lacZ (C) fusions. Cells were grown in LD medium. β-Galactosidase specific activity (Continued on next page)
activated by LptC depletion (Fig. 4A). We also observed ldtD but not ldtE activation in wild-type BW25113 cells carrying pldtD-lacZ or pldtE-lacZ and treated with LPC-058 (Fig. 3C).

In summary, ldtE and ldtF are housekeeping LDTs which share a growth phase-dependent activation profile under all conditions tested, and their expression was unaffected by the presence or absence of arabinose in the araB/pLptC conditional strain. In contrast, ldtD was strongly expressed in the lptC+ background in which both ldtE and ldtF were deleted, in LptC-depleted cells, and in cells with blocked LPS synthesis. Hence, LdtD is the stress LDT activated under cell envelope stress conditions or in the absence of the housekeeping LdtE/LdtF, consistent with the presence of increased levels of 3-3 cross-links under these conditions.

Growth arrest without lysis requires PG synthesis and maturation. Thus far, our data suggest that LDTs play a major role in PG remodeling in protecting cells from lysis upon LPS export pathway defects. LDTs can facilitate PG growth in certain β-lactam-resistant strains of E. coli and E. faecium, and in this situation, they function with a GTase domain of a bifunctional PG synthase, and a DD-CPase (42–45). LptC-depleted cells have been shown previously to have elevated levels of the bifunctional PBP1B and the DD-CPases PBP5 and PBP6a (46). PBP5 is the major DD-CPase active under standard laboratory conditions (32). PBP6a is an additional DD-CPase with an unknown physiological function, as it does not seem to be active under standard growth conditions (35).

We next asked whether bifunctional PBPs and DD-CPases are important to prevent lysis in LptC-depleted cells, as are the LDTs. PBP1B, but not PBP1A, was required to prevent lysis of LptC-depleted cells (Fig. 5A to D), and lysis could be prevented by ectopic expression of PBP1B (Fig. 5E). We next tested which of the two activities of PBP1B was needed to prevent lysis. The ectopic expression of PBP1B(S510A) with an active GTase and inactive TPase domain was fully functional in preventing lysis, showing that the TPase activity of PBP1B is not required (Fig. 5E). However, the ectopic expression of PBP1B(E233D) with inactive GTase function was unable to prevent lysis of LptC-depleted cells lacking wild-type PBP1B, suggesting that the GTase activity of PBP1B is crucial to prevent lysis (Fig. 5E). Consistent with this conclusion, lysis was also observed in cells lacking LpoB, a key activator of the GTase of PBP1B (26, 28, 50) (Fig. S4C and D). Another regulator of PBP1B, CpoB (27), was not required to prevent lysis upon LptC depletion (Fig. S4E), consistent with CpoB’s exclusive regulation of the TPase function of PBP1B and our findings that TPase was not needed to prevent lysis.

Finally, survival of LptC-depleted cells required the DD-CPase gene dacC, encoding PBP6a, but not the dacA gene encoding PBP5 (Fig. 6). Therefore, preventing lysis upon severe LPS transport defect requires not only LDTs but also the GTase activity of PBP1B and the DD-CPase PBP6a, presumably to synthesize and to modify the nascent PG substrate for the LDTs.

LdtD interacts with PBP1B. Our data supported the hypothesis that LdtD may function with PBP1B to rescue sacculus integrity upon severe OM assembly defects. We then asked whether LdtD physically interacts with class A PBPs by mixing purified

FIG 4 Legend (Continued)
oligohistidine-tagged PBP1A or PBP1B with untagged LdtD and assaying binding to Ni²⁺-NTA beads. LdtD was pulled down by oligohistidine-tagged PBP1B, not by oligohistidine-tagged PBP1A or LpoB, or in the absence of tagged protein (Fig. 7A), suggesting a direct interaction with oligohistidine-tagged PBP1B. The pulldown was confirmed and extended by microscale thermophoresis, which revealed an interaction between LdtD and PBP1B, but not between LdtD and PBP1A. The $K_d$ value of the LdtD-PBP1B interaction was $112 ± 33$ nM (Fig. 7B). Moreover, PBP1B was pulled down by oligohistidine-tagged LdtD, expressed from the chromosome from its native promoter, only upon LptC depletion (Fig. 7C), and LdtD and PBP1B interacted in LptC-depleted cells as shown by chemical cross-linking followed by immunoprecipitation (Fig. 7D). These data suggest that a PBP1B-LdtD complex is formed in cells experiencing an OM assembly defect.
The LDT activity of LdtD has been demonstrated previously with a soluble disaccharide tetrapeptide substrate (45). Considering its role in PG remodeling and its interaction with PBP1B, we hypothesized that the enzyme must be active against larger PG fragments or even sacculi and/or nascent PG produced by PBP1B. We tested these possibilities by first incubating LdtD with either soluble glycan chains carrying non-cross-linked tetrapeptides (DS-tetra chains, the products of MepM [Fig. 8A]) and PG sacculi purified from strain BW25113ΔLDT. LdtD was highly active against these substrates (Fig. 8A), utilizing almost all monomeric tetrapeptides to generate the 3-3 cross-linked dimer (disaccharide tetratripeptide, TetraTri). The high activity is particularly remarkable in the case of the sacculi, which after the reaction with LdtD contained an unusually high cross-linkage with ~84% of all muropeptides present in cross-links.

We next assayed the activity of LdtD during synthesis of PG in vitro using radiolabeled lipid II as the substrate in the presence of 10-fold excess of unlabeled PG sacculi.
After the reaction, the products were digested with the muramidase cellosyl, and the resulting muropeptides were separated by HPLC using back-to-back UV and radioactivity detectors to monitor the products formed. LdtD produced a highly 3-3 cross-linked nascent PG, as seen by the abundant radiolabeled TetraTri(3-3) muropeptide present in the reaction with the TPase-inactive PBP1B (S510A) mutant, its activator LpoB, and the DD-CPase PBP6a (red trace in sample III [Fig. 8B]). In the absence of LdtD, PBP1B (S510A)/LpoB produced non-cross-linked glycan chains with pentapeptides of which most were trimmed by PBP6a to tetrapeptides (red traces in sample II [Fig. 8B]), and no 3-3 cross-links were observed in the UV traces (sample III [Fig. 8B]). Remarkably, LdtD preferentially acted on the nascent (radioactive) PG (red trace, sample III) despite the presence of a 10-fold excess of unlabeled PG sacculi (black trace, sample III). The UV traces showed that ~52% of the unlabeled tetrapeptides were consumed by LdtD (comparing the black traces in samples II and III [Fig. 8B]), which was markedly less than

FIG 7 LdtD interacts with PBP1B in vitro and in vivo. (A) Coomassie blue-stained SDS-PAGE gel showing the pulldown of proteins to Ni\(^{2+}\)-NTA beads. LdtD bound to the beads and was present in the elution fraction (lanes E) only in the presence of oligohistidine-tagged PBP1B, and not in the presence of oligohistidine-tagged LpoB, oligohistidine-tagged PBP1A, or in the absence of another protein. A, applied sample. (B) Microscale thermophoresis curves showing that LdtD interacts with PBP1B and not with PBP1A. The \(K_D\) value for the LdtD-PBP1B interaction is indicated. Values are means ± SD from three independent experiments. (C) BW25113 ldtD-his and araB plptC ldtD-his cells grown with and without arabinose (Ara) were treated with the DTSSP cross-linker. Cell-free extract was prepared, and LdtD-His was purified onto a Ni-NTA resin. PBP1B, LdtD-His, and LptE (as loading control) were immunodetected after SDS-PAGE and Western blotting. WT, wild type. (D) In vivo interaction between PBP1B and LdtD-His by cross-linking/coimmunoprecipitation assay. araB plptC ldtD-his cells were treated with cross-linker DTSSP. The membrane fraction was prepared, and PBP1B was precipitated by specific antibody (the control sample received no antibody). LdtD-His was detected by Western blotting using specific anti-oligohistidine-tag antibody. M, membrane extract; S, supernatant; E, elution.
the ~68% consumption of the radiolabeled tetrapeptides (comparing the red traces in samples II and III [Fig. 8B]). This suggests that LdtD prefers new PG, synthesized by PBP1B and trimmed by PBP6a, as the substrate. LdtD showed similar activity in reactions with PBP5 (instead of PBP6a), showing that both DD-CPases are capable of providing the tetrapeptide substrates (Fig. S6).

Together, the results of the activity assays support the phenotypic data and muropeptide analysis showing that LdtD is highly active in producing 3-3 cross-links in PG sacculi, and it is able to cooperate with the GTase activity of PBP1B and DD-CPases to utilize nascent PG as the substrate, consistent with a role in protective remodeling of PG during OM defective assembly.

**DISCUSSION**

LPS is essential in many Gram-negative bacteria with several notable exceptions, namely *Neisseria meningitidis* (51), *Moraxella catarrhalis* (52) and *Acinetobacter baumanii* (53), which can grow without LPS. *E. coli* requires LPS, and therefore, the depletion of LptC is not compatible with cell growth. However, although cells are unable to
continue growing, they do survive the block of LPS transport for several hours, and they resume growth once expression of LptC is restored.

In this work, we discovered a PG remodeling pathway involving LDTs that is essential for survival in cells with defective OM assembly, revealing a link between LPS export and a dedicated mode of PG synthesis. LDTs are not required in unstressed cells which, however, do remodel the PG to introduce a small number of 3-3 cross-links upon entry into stationary phase, perhaps to repair minor defects in PG. Expanding from previous work (39–41), we also show here that E. coli has an additional YkuD homologue, LdtF, which is not an active LD-TPase per se but might stimulate other LDTs.

**Roles of the different LDTs.** LdtE is the housekeeping LDT that is induced by RpoS when cells enter stationary phase (Fig. 4) consistent with the increase in 3-3 cross-links in stationary-phase cells (36, 54). LdtE seems to require LdtF for activity (Fig. 2) and the LdtE-LdtF couple forms most of the 3-3 cross-links in unstressed cells in which LdtD is poorly expressed (Table 1 and Fig. 4).

LDTs become essential to prevent cell lysis in LptC-depleted cells which upregulate ldtD and increase 3-3 cross-links (Fig. 4 and Table 1). Notably, LDTs are inhibited by sub-MIC copper ions which therefore reduce the robustness of the cell envelope to withstand LPS export stress (55). That LdtD plays a major role in PG remodeling during cell envelope stress is consistent with its induction by the Cpx-mediated stress response (56, 57). The single ΔldtE or ΔldtF mutants lysed upon LptC depletion despite the presence of a functional copy of ldtD; presumably, they are unable to accumulate sufficient LdtD activity to avoid lysis upon LptC depletion. In contrast, the ΔldtE ΔldtF double mutant is already stressed and has a high level of LdtD (and of 3-3 cross-links) before the depletion of LptC, preventing lysis once LptC is depleted (Table 1). This conclusion is further supported by the finding that ectopic expression of ldtD prevents lysis of all single and multiple ldt mutants depleted for LptC. For unknown reason, the ldtF mutant shows impaired cell morphology even before LptC depletion (see Fig. S3D in the supplemental material), suggesting that enhanced 3-3 cross-links are not always protective and that LdtF, which has been implicated in biofilm formation in enteroaggregative E. coli (58), has an additional role in the cell. Hence, our PG analysis highlights that an increased level of 3-3 cross-links cannot protect every ldt mutant cell from lysis, but importantly, the ability of cells to avoid lysis is always accompanied by an increase in 3-3 cross-links (Table 1).

**LdtD is part of a “PG repair machine” with PBP1B/LpoB and PBP6a.** LptC-depleted cells also required the GTase function of PBP1B, its activator LpoB, and the DD-CPase PBP6a (but not PBP1A or PBP5) to avoid lysis (Fig. 5 and 6). To our knowledge, this is the first condition where PBP6a becomes important. Our genetic evidence (Fig. 5C and D), the previously observed induction of the PBP1B and PBP6a genes in LptC-depleted cells (46), and the physical interaction of LdtD with PBP1B in vitro and in stressed cells (Fig. 7) all support a model in which PG remodeling machinery containing PBP1B/LpoB, LdtD, and PBP6a polymerizes PG strands (GTase of PBP1B), trims the pentapeptides (PBP6a), and utilizes the resulting tetrapeptides to form 3-3 cross-links (LdtD) (Fig. 9).

**How does PG remodeling rescue cells from lysis?** The PG layer is an elastic, net-like structure thought to be the major stress-bearing structure in the bacterial cell envelope allowing the cell to sustain large mechanical loads such as turgor pressure (37, 59). This prevailing dogma has been challenged by studies of phage lysis (60, 61) and more recently by Rojas and coworkers who showed that the OM and PG balance the mechanical loads during osmolality changes (62). Interestingly, a mutant defective in LPS export carrying the imp4213 allele of lptD (63) produced an OM with an altered load-bearing capacity (62). Defects in the OM (i.e., perturbation of LPS layer and local loss of lipid asymmetry) may cause local mechanical stress on the PG structure, and hence, the LdtF-mediated PG remodeling could strengthen the PG to rebalance the mechanical load between the OM and cell wall.

We also envision another possible reason why LDTs are essential upon defective LPS export. The size of pores in the PG net is too small for large transenvelope assemblies,
such as the flagella and type II secretion systems, and hence, the assembly of these requires the local hydrolysis of the PG to increase the pore size (64, 65). The width of the periplasmic Lpt “bridge” together with its bulky LPS cargo (19, 66) is likely wider than the diameter of pores in PG (4.1 to 6.2 nm, depending on the turgor), necessitating the local hydrolysis of the PG, by an as yet unknown PG hydrolase, for the assembly of the Lpt machinery and rapid flux of LPS to the cell surface. The Lpt complex is known to disassemble when LPS transport is arrested due to depletion of LptC (14, 17, 18). Hence, it is possible that LDTs seal (repair) the PG locally after the disassembly of LPS export machines. We propose a dedicated PG repair machine, containing PBP1B/LpoB, LdtD, and PBP6a for this function (Fig. 9). The sequence of events that lead to cell lysis following the block of LPS biogenesis in the absence of LDTs are currently not known, but our data suggest that lysis is likely the consequence of the accumulation of defects in the PG that cannot be repaired and lead to unbalanced mechanical load between the OM and PG.

The GTase function of the PG repair machine is activated by the OM-anchored lipoprotein LpoB, which spans the periplasm to interact with the UB2H domain of PBP1B. Hence, apart from its role in the synthesis of “normal PG” (with 4-3 cross-links) during cell elongation and division, the PBP1B-LpoB system has another role in PG remodeling together with LdtD, producing PG with 3-3 cross-links. PBP1B/LpoB, LdtD, and the DD-CPase PBP5 enabled an *E. coli* mutant strain to grow in the presence of an otherwise lethal concentration of ampicillin (45), and PBP1B/LpoB (and not PBP1A/LpoA) promoted the recovery of PG-less L-form cells of *E. coli* to the walled state, generating a PG layer *de novo* (67). These observations and our own work highlight the versatility of the PBP1B/LpoB PG synthase/regulator pair, which is used by the cell in different processes and circumstances. PBP1A/LpoA are able to compensate for the loss of PBP1B/LpoB in normal growth, but they cannot compensate for the stress-related function of PBP1B/LpoB with LptD. Indeed, cells in which PG synthesis may be considered “weakened” by the lack of PBP1A or PBP5 do survive LPS transport defects just as well as wild-type cells. Hence, our combined data support a specific PG remodeling mechanism instead of nonspecific effects such as a general “weakening” of PG synthesis.

In summary, we discovered a role of 3-3 cross-links in the PG as a mean to fortify the sacculus in response to severe OM assembly defects. This functional connection between OM biogenesis and PG remodeling highlights an elegant and versatile mec-
anism bacteria employ to maintain the integrity of their essential cell envelope under a variety of growth and stress conditions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. Primers used are listed in Table S2. Cells were routinely grown aerobically at 37°C or 30°C in LB-Lennox medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) (Difco). When required, antibiotics or inducers were added: ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (25 μg/ml), arabinose (0.2% [wt/vol]), IPTG (0.1 mM). For LptC depletion, bacteria were harvested from cultures with an OD_{600} of 0.2 by centrifugation, washed twice with LD, and diluted 100-fold in LD with or without arabinose. Cell growth was monitored by OD_{600} measurements, and viability was determined by quantifying the colony-forming units (CFU).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02729-18.

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