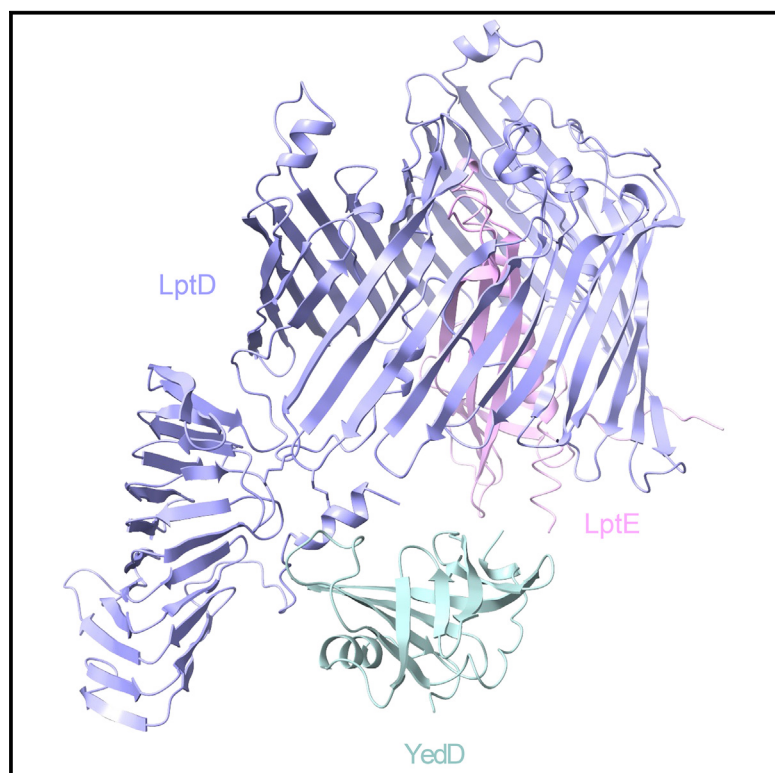


Optimal functioning of the Lpt bridge depends on a ternary complex between the lipocalin YedD and the LptDE translocon

Graphical abstract



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In brief

Gennaris et al. identify YedD as a component of the Lpt system in *Escherichia coli*. Cryo-EM analysis reveals YedD interacting with the outer membrane LptDE translocon, with its binding proposed to stabilize the Lpt bridge. Deletion of *yedD* exacerbates LPS transport defects, highlighting its critical role in optimizing Lpt function.

Highlights

- Cryo-EM structure reveals YedD interaction with the LptDE translocon
- YedD stabilizes the Lpt bridge and optimizes LPS transport
- *yedD* deletion impairs Lpt function under bridge-destabilizing conditions
- YedD's function links lipocalins to outer membrane biogenesis



Article

Optimal functioning of the Lpt bridge depends on a ternary complex between the lipocalin YedD and the LptDE translocon

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SUMMARY

The outer membrane is an efficient permeability barrier that protects gram-negative bacteria against external assaults, including many antibiotics. The unique permeability features of the outer membrane are due to the presence of lipopolysaccharide (LPS) molecules in its outer leaflet. LPS transport relies on the essential lipopolysaccharide transport (Lpt) pathway, which forms a bridge from the inner to the outer membrane. The LptDE translocon inserts LPS into the outer leaflet. Here, we identify the lipocalin YedD as a component of the translocon. Cryoelectron microscopy of the YedD-LptDE complex reveals that YedD binds LptD at a critical interface between its β -barrel and periplasmic β -taco domain. The YedD-LptDE complex is functionally relevant: under conditions where the connectivity of the β -taco and Lpt bridge is compromised, the absence of YedD decreases cell viability and causes LPS accumulation in the inner membrane. Our findings establish YedD as an Lpt component required for optimal LPS transport.

INTRODUCTION

The hallmark of gram-negative (diderm) bacteria is their cell envelope, which is composed of two membranes confining the aqueous periplasm in which the peptidoglycan cell wall lies.^{1–3} While the inner membrane is a classical phospholipid bilayer that delineates the cytoplasmic space, the outer membrane is an asymmetric membrane with phospholipids in the inner leaflet and lipopolysaccharides (LPSs) in the outer leaflet, facing the extracellular environment. LPS is a large glycolipid that is organized in 3 regions: (1) the hydrophobic lipid A that anchors LPS into the outer membrane and is made of a di-phosphorylated disaccharide decorated with 4–7 acyl chains, (2) the core, branched oligosaccharide region that contains about 10 sugars, some of which are phosphorylated, and (3) a highly variable polysaccharide chain known as the O-antigen, a major virulence determinant.^{3,4} The strong lateral interactions between the acyl chains of lipid A, together with the bridging of the negatively charged phosphates of adjacent LPS molecules by divalent cations (Ca^{2+} and Mg^{2+}), render the outer membrane poorly permeable to a large array of molecules, including many antibiotics that are active against gram-positive (monoderm) bacteria. Furthermore, recent studies have revealed that the outer membrane also plays a significant mechanical role as a load-bearing

structure that is required for building up periplasmic turgor under hypoosmotic shocks.^{5,6} The outer membrane composition, asymmetry, and LPS crosslinking are essential for its function as a mechanical support and physicochemical barrier.^{1–4,7–9} Thus, the timely delivery of outer membrane lipids and the proper assembly and maintenance of the outer membrane are crucial for growth and survival.

The synthesis of LPS molecules begins at the cytoplasmic leaflet of the inner membrane, where the core oligosaccharide is attached to the lipid A moiety, forming lipooligosaccharide molecules.^{3,4} Lipooligosaccharides are then translocated to the periplasmic leaflet of the membrane by the ABC transporter MsbA, before the WaaL ligase covalently attaches the O-antigen to the core oligosaccharide, yielding LPS molecules. Transport of LPS to the outer membrane depends on the *trans*-envelope lipopolysaccharide transport (Lpt) system (Figure 1A), which is composed of 7 essential proteins (LptABCDEFG).³ After LPS extraction from the inner membrane by the ABC transporter LptB₂FGC, the glycolipid is positioned on the *trans*-envelope bridge formed by the head-to-tail association of the structurally homologous β -taco folds of LptF, LptC, LptA, and LptD, together forming a continuous C-shaped hydrophobic groove that shields the acyl chains of LPS from the aqueous periplasm.^{10–13} It is then thought that multiple rounds of ATP binding and hydrolysis



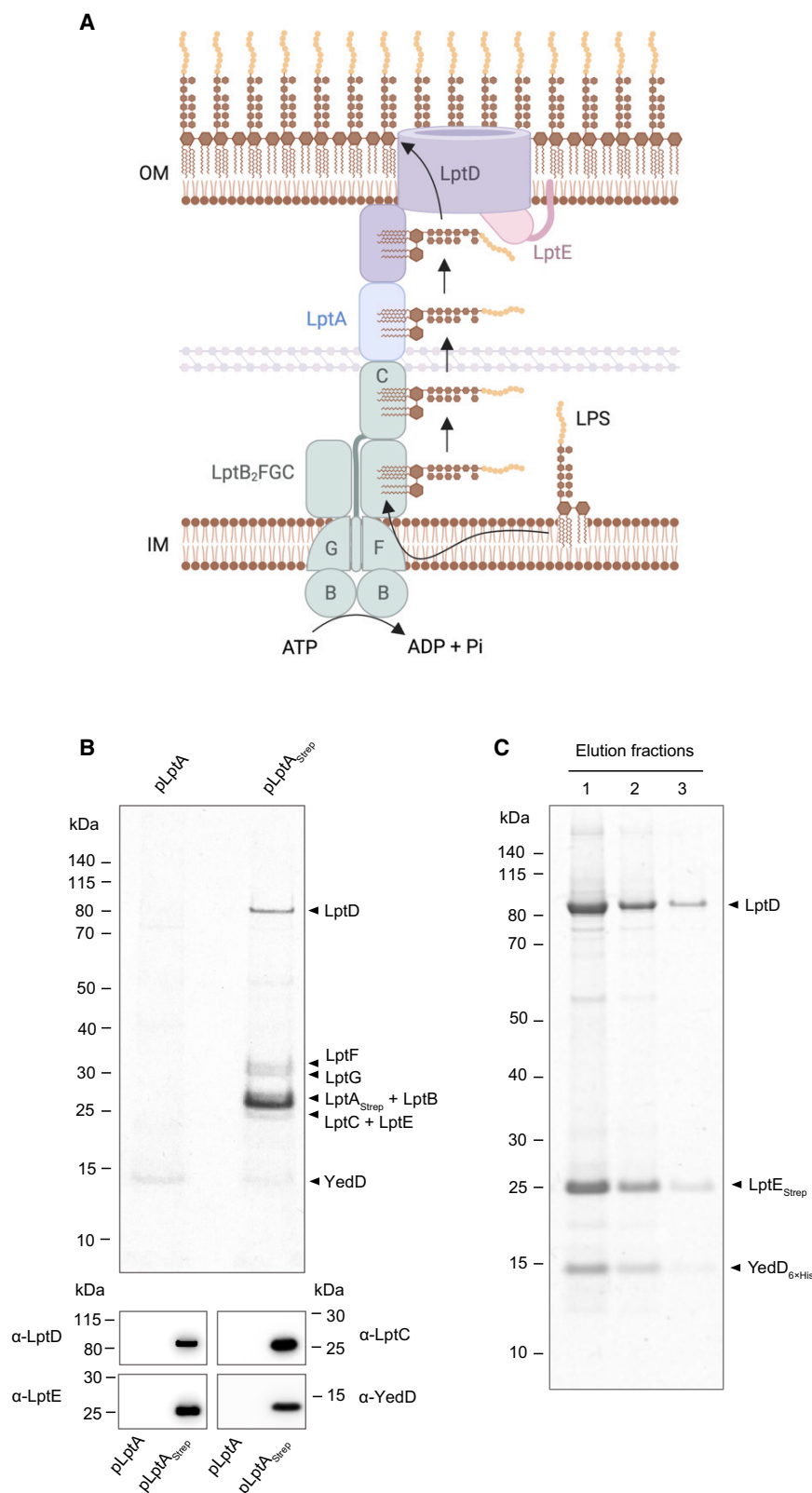


Figure 1. YedD co-purifies with the Lpt trans-envelope bridge and forms a stable complex with the LptDE translocon

(A) A schematic (created with [BioRender.com](#)) showing all 7 components of the Lpt transport machinery and the movement of LPS across the envelope.

(B) Top, Strep-Tactin affinity purification was performed on total membrane extracts of a $\Delta lptA::kan$ strain expressing either LptA without a tag (as a control; pLptA) or a Strep-tagged LptA (pLptA_{Strep}) at low levels from the pET23/42 plasmid. Pull-downs were followed by SDS-PAGE analysis and protein staining. Proteins co-eluting with LptA_{Strep} were identified by band excision and MS analysis. Bottom, immunoblot analysis of samples shown on the top using antibodies directed toward LptD, LptE, LptC, and YedD.

(C) SDS-PAGE analysis of three successive elution fractions obtained after the second of two consecutive affinity purification steps, first using LptE_{Strep} and then YedD_{6xHis} as bait proteins, following co-expression of the LptDE translocon containing a Strep-tagged LptE (LptE_{Strep}) and a His-tagged YedD (YedD_{6xHis}).

The images shown in (B) and (C) are representative of three independent experiments. See also [Figure S2](#).

push LPS molecules in a continuous stream across the bridge until they reach the heterodimeric LptD-LptE translocon responsible for the assembly of the glycolipid at the outer membrane (Figure 1A).^{14,15}

LptD is composed of 2 domains: an N-terminal domain with a β -taco fold and a large, 26-stranded C-terminal bilobed β -barrel domain (Figure S1A). Two non-consecutive disulfide bonds (C31–C724 [C1–C3] and C173–C725 [C2–C4]; Figure S1B) connect the N-terminal to the C-terminal domain.^{13,16,17} The presence of at least one of these disulfides is required for LptD to be functional and ensure cell viability, suggesting that disulfide formation is crucial for the correct positioning of the N- and C-terminal domains of LptD relative to one another.^{16–18} LptE is a lipoprotein that plays a crucial role by both enabling LptD to acquire its native conformation and serving as a plug in the barrel of the mature translocon by deeply inserting into one of the lobes of the LptD β -barrel (Figures S1A, S1C, and S1D).^{13,16,17,19–21} Additionally, LptE also assists LptD in inserting LPS molecules into the outer membrane.^{13,22} Structural and functional studies have revealed that the junction between the β -taco and β -barrel domains as well as the interface between the first and last strands of the β -barrel are crucial for function.^{13,16,17,21,23–25} The current model is that LPS acyl chains are inserted into the membrane by exiting an intra-membrane hole at the distal tip of the β -taco domain, thus driving the rest of the molecule to pass through a luminal gate at the base of the barrel domain before entering the lumen of LptD in the lobe not occupied by LptE.^{13,16,17,21,23–25} The luminal gate is formed by loop 1 (L1) and L2; L1 connects the first β strand of the barrel to the N-terminal domain, while L2 links the last β strand of the barrel to the LptD C terminus (Figure S1E). The first and last strands of the barrel form a putative lateral gate to the membrane. Opening of the luminal gate by the passing substrate is thought to act as a switch triggering the opening of the β -barrel, allowing lateral exit of the hydrophilic part of the LPS molecule into the outer leaflet of the outer membrane following its vertical reorientation in the lumen.^{17,23,24}

However, despite a significant amount of evidence that provided important insights into the transport of LPS molecules across the envelope,³ several questions remain unanswered regarding how large amphipathic LPS molecules transition from the *trans*-envelope bridge to the outer membrane and become inserted in the outer leaflet; crucial mechanistic details on this process are still lacking, in part due to the absence of LPS molecules in solved LptDE structures.^{13,16,17,21,25} Furthermore, recent data suggest that the activity of the Lpt system is regulated to prevent the outer membrane from becoming overloaded with glycolipid²⁶ or to assemble the *trans*-envelope bridge only if the LptB₂FGC transporter or the LptDE translocon is functional.^{11,27} However, our understanding of Lpt regulation remains limited. Due to the complexity of LPS transport and the probable necessity of regulating the process for optimal functioning, we hypothesized that accessory components of the Lpt bridge involved in the functioning of the system remained to be identified. Here, we report the identification of YedD, an outer membrane lipoprotein and a member of the ubiquitous lipocalin family,^{28,29} as a component of the Lpt system. We discovered that YedD interacts with the LptDE translocon. Using cryoelectron microscopy (cryo-EM) to elucidate the structural details

of the LptDE-YedD ternary complex, we observed that YedD associates with the hinge region between the N- and C-terminal domains of LptD. Investigating the impact of *yedD* deletion on Lpt function, we acquired molecular evidence indicating that YedD is essential for proper LPS transport to the outer membrane, stabilizing the Lpt bridge and optimizing LptDE activity.

RESULTS

The lipocalin YedD binds the LptD-LptE translocon

To identify molecular factors regulating Lpt activity, we aimed to characterize the interactome of LptA, the periplasmic component of the Lpt bridge. LptA, fused to a C-terminal Strep-tag (LptA_{Strep}), was expressed at low levels and affinity purified (Figure 1B). By conducting mass spectrometry (MS) analysis of the co-eluting bands, we identified the six other members of the Lpt bridge, as expected (Figures 1B and S2).³⁰ Remarkably, we found that LptA_{Strep} also co-purified with YedD, an outer membrane lipoprotein of unknown function belonging to the lipocalin family (Figures 1B and S2).^{28,29} Lipocalins, which are found across a wide range of species, share a distinct three-dimensional (3D) structure. This structure consists of an elliptical eight-stranded antiparallel β -barrel creating a hydrophobic cavity, connected to a short N-terminal 3_{10} helix and a C-terminal α helix. The C-terminal α helix is positioned against the β sheet, and in some cases, such as in YedD, it is stabilized by a disulfide bond (Figure S2).^{28,29} Several eukaryotic lipocalins have been characterized, providing evidence that these proteins predominantly bind small, hydrophobic ligands and play pivotal roles in a diverse range of biological processes.^{28,29,31} For instance, retinol-binding protein transports vitamin A in human plasma through its deep and narrow cavity,³² while neutrophil gelatinase-associated lipocalin, secreted during immune activation, binds siderophores to inhibit bacterial growth.³³ However, not all lipocalins bind hydrophobic molecules. Human glycodefin, for example, lacks a deep hydrophobic cavity in its β -barrel. Instead, it forms a homodimer with glycosylation sites strategically exposed on one surface, enhancing its avidity for cellular receptors involved in reproduction processes.³⁴ The depth of YedD's cavity is comparable to that of glycodefin.

Considering the targeting of YedD to the outer membrane, we postulated that it might specifically interact with the LptDE translocon. To verify this, we co-expressed LptDE (in which LptE was fused to a C-terminal Strep-tag [LptE_{Strep}]) and YedD (fused to a C-terminal His-tag [YedD_{6xHis}]) and performed two successive affinity purification steps, first using LptE_{Strep} and then YedD_{6xHis} as bait. This strategy resulted in the purification of a stable, stoichiometric ternary complex consisting of YedD and LptDE, thereby confirming the interaction of YedD with the LptDE translocon (Figure 1C).

YedD binds a region of the LptDE translocon that is essential for function

Intrigued by this result, we sought to obtain structural information on the LptDE-YedD complex using cryo-EM 3D single-particle reconstruction, resulting in a 3.89 Å electron potential map that allowed the unambiguous building of the model (Figure S3 and Table S1). As shown in Figure 2A, YedD binds LptD to the

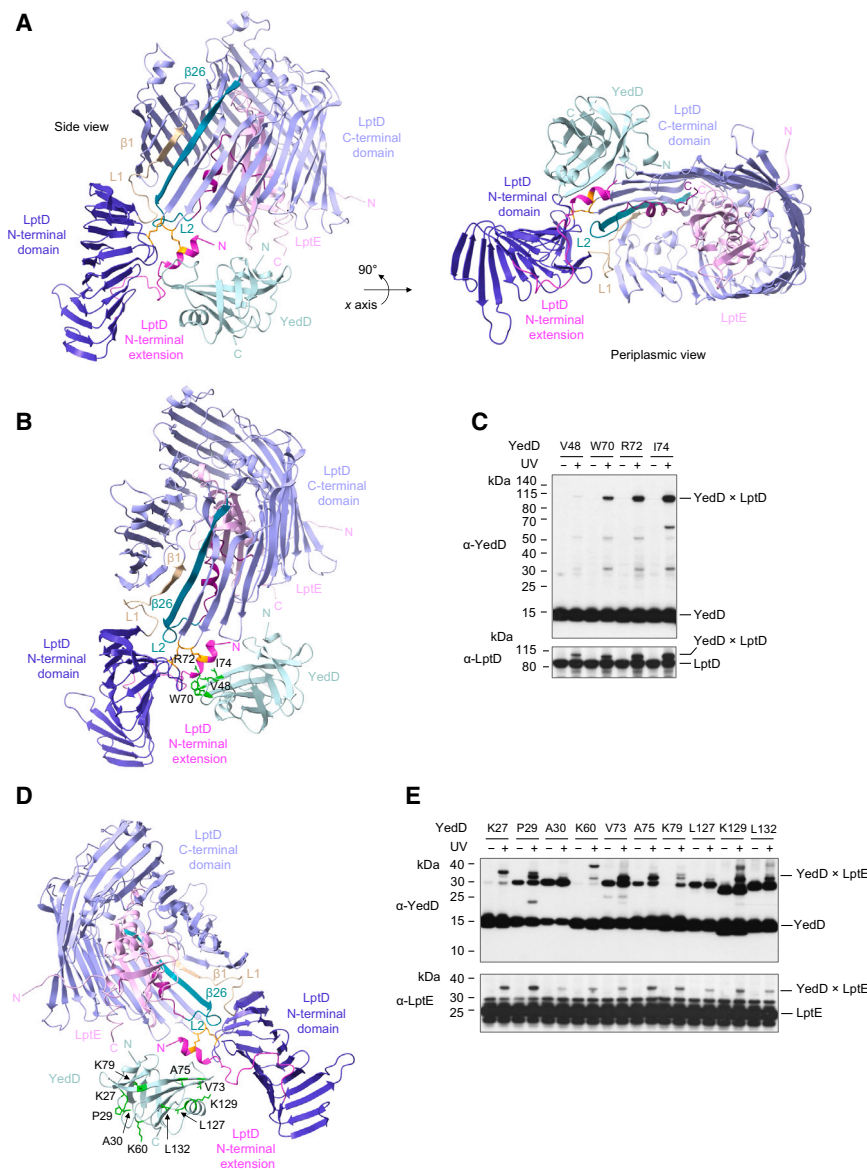


Figure 2. YedD interacts with the N-terminal domain of LptD and with LptE

(A) Side (left) and periplasmic (right) ribbon diagram views of the LptDE translocon in complex with YedD. The LptD C-terminal domain is represented in light blue and the N-terminal domain in dark blue, with the N-terminal extension shown in magenta. The two non-consecutive disulfide bonds present in LptD are shown as orange sticks. Luminal loop 1 (L1) and the β strand 1 (β 1) that follows it are shown in sand, whereas luminal L2 and the β 26 that precedes it are shown in teal. The C-terminal extension of LptD immediately after L2 is depicted in purple. LptE is represented in light pink and YedD in pale cyan. N and C represent the N and C termini, respectively, of LptD, LptE, and YedD.

(B) View of the interaction interface of YedD with LptD. Amino acids shown as green sticks were substituted with pBpa for experiments shown in (C). (C) YedD containing pBpa at the indicated positions was photo-crosslinked *in vivo*, and adducts were detected by immunoblotting with antibodies directed toward YedD and LptD. The additional bands recognized by the anti-YedD antibodies likely represent crosslinks between YedD and as-yet-unidentified proteins, while the band at ~30 kDa corresponds to a YedD dimer.

(D) View of the interaction interface of YedD with LptE. Amino acids shown as green sticks were substituted with pBpa for experiments shown in (E). There is no cryo-EM density for the LptE C-terminal residues 169–193, which are predicted to be part of a disordered region. These residues have been modeled and are shown in Figure S5.

(E) YedD containing pBpa at the indicated positions was photo-crosslinked *in vivo*, and adducts were detected by immunoblotting with antibodies against YedD and LptE. The YedD-LptE crosslinks are indicated. The additional bands recognized by the anti-YedD antibodies likely represent crosslinks between YedD and as-yet-unidentified proteins, while the band at ~30 kDa corresponds to a YedD dimer.

The images shown in (C) and (E) are representative of three independent experiments. See also Figures S1–S4 and Table S1.

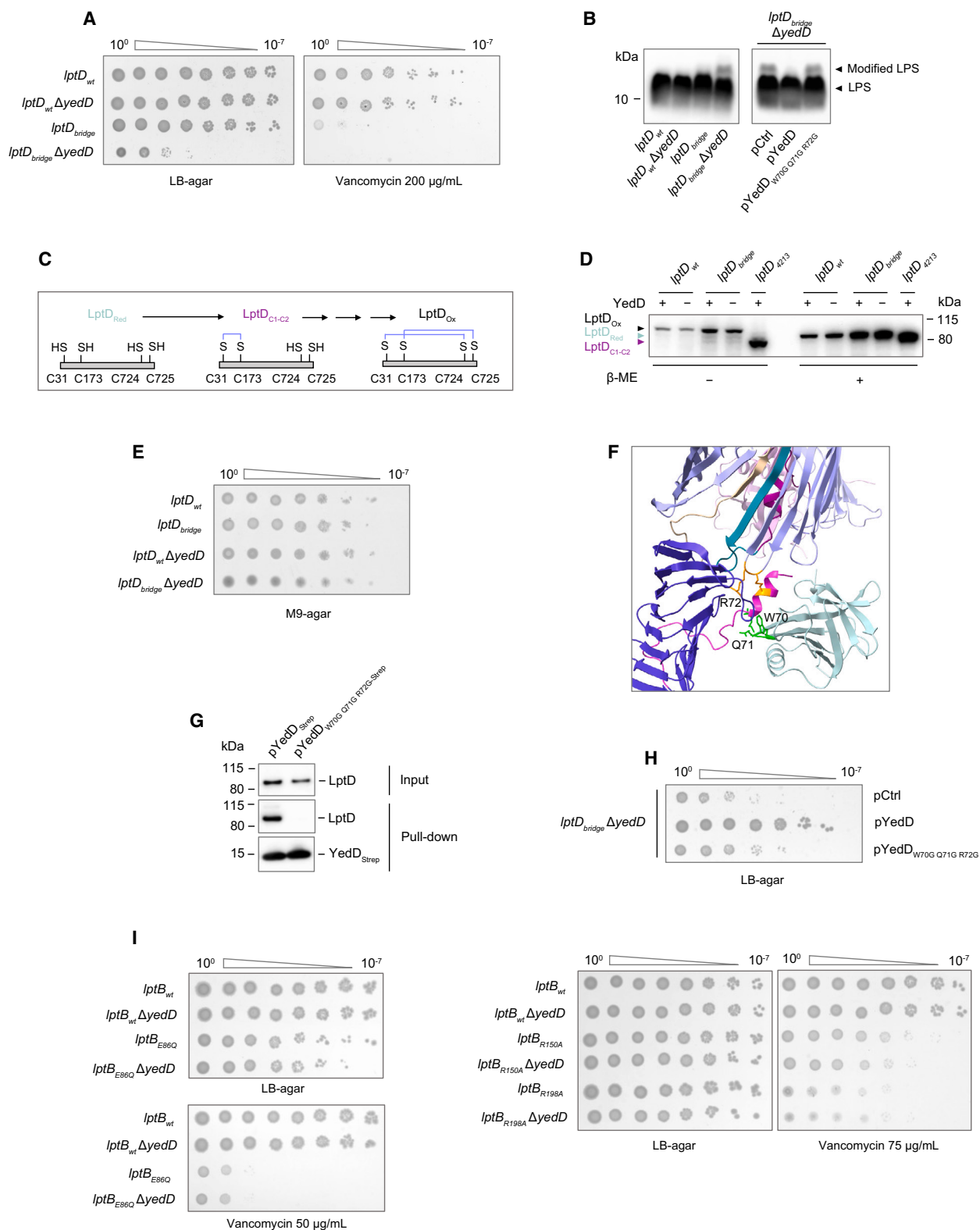
hinge region between the N-terminal periplasmic β -taco and the C-terminal β -barrel domains. The LptD β -barrel in the structure assumes a closed conformation, consistent with previously reported structures (Figure S4).^{13,17,21} Like in *Klebsiella pneumoniae* LptD, the β -taco domain rotates away from the β -barrel, and the luminal gate loops assume a more open position (Figure S4).¹⁷ Regarding YedD, it retains its characteristic lipocalin architecture when it forms a complex with LptDE (Figures 2A, 2B, 2D, and S4).

In full-length enterobacterial LptDE structures, the N-terminal domain of LptD contains a flexible extension of about 30 residues (residues 25–52 in *E. coli* LptD) at its N terminus. This extension folds back along the periplasmic β -taco domain (Figures 2A and S1)^{13,17} and is covalently connected to the β -barrel domain via a disulfide bond (C1–C3, see below); it also contains a short α helix, which forms close contacts with the interface between the

β -taco and β -barrel domains, establishing interactions with L2 of the luminal gate (Figure S1). Strikingly, in our structure, we see that the short N-terminal α helix binds the hydrophobic cavity of YedD (Figures 2A and S2); YedD is thus positioned close to a region of the LptDE translocon that is essential for function. In addition, YedD does not seem to occlude the path likely taken by LPS molecules to reach the outer leaflet of the membrane.

Site-specific photo-crosslinking extends the binding interface to the disordered region of LptE

To validate the interaction interface between YedD and LptDE, we modified a plasmid-borne *yedD* allele to produce variants of the lipoprotein. These variants were substituted with the photo-crosslinkable unnatural amino acid pBpa³⁵ at multiple sites throughout the 3D structure (Figures 2B and 2D). Upon expression of the *yedD* variants and UV exposure, numerous



(legend on next page)

crosslinks between YedD and both LptD and LptE were detected (Figures 2C and 2E). In line with the structural data, we observed strong *in vivo* photo-crosslinking between LptD and YedD when pBpa was inserted at positions V48, W70, R72, and I74 in YedD. These positions are all located either at the entrance or inside the hydrophobic cavity (Figures 2B, 2C, and S2). We also detected crosslinks between YedD and LptE when pBpa was introduced in YedD at positions K27, P29, A30, K60, V73, A75, K79, L127, K129, and L132. These positions are all located on the surface of YedD (Figures 2D, 2E, and S5). Because the structure does not show YedD interacting with LptE, it is likely that the detected YedD-LptE interaction involves the disordered¹⁷ C-terminal region of LptE (residues 169–193), which is supported by molecular dynamics simulations (Figures 2A, 2D, and S5).

Formation of the LptDE-YedD complex is required for optimal LPS transport by the Lpt bridge

Unlike the previously identified components of the Lpt bridge, YedD is not essential.³⁶ However, the fact that YedD forms stable complexes with the LptDE translocon, interacting with a region of LptD that is critical for the translocation of LPS molecules to the outer leaflet of the outer membrane, suggested that YedD is involved in the functioning of the Lpt system. We decided to explore this hypothesis by testing whether mutations in functionally important regions of LptD become toxic in a *yedD* deletion background.

First, we introduced mutations in the region of LptD that is important for interactions with the periplasmic component LptA. To destabilize the bridge, two residues that are at the interface between LptD and LptA (D57 and K60) were deleted, generating LptD_{bridge} (Figure S5F).^{11,37} As shown by others, cells ex-

pressing LptD_{bridge} grow like the wild type but are sensitive to the large hydrophilic antibiotic vancomycin due to defects in LPS transport (Figure 3A).³⁷ Interestingly, we found that deleting *yedD* in these cells resulted in a severe growth defect, even in the absence of vancomycin (Figure 3A). Moreover, the growth defect of *lptD_{bridge} ΔyedD* cells correlated with the accumulation of an anomalous form of LPS migrating more slowly than native LPS (Figure 3B). The accumulation of modified LPS molecules occurs upon a severe decrease in LPS transport to the outer membrane.^{38,39} In this case, newly synthesized glycolipid molecules accumulate at the inner membrane, resulting in their aberrant modification with colanic acid by the WaaL O-antigen ligase,^{38,39} which is located in the inner membrane. We verified that the growth defect and the accumulation of modified LPS in the *lptD_{bridge} ΔyedD* cells did not result from improper oxidative folding of LptD_{bridge} nor a decrease in its steady-state levels (Figures 3C and 3D). To assess the oxidative folding of LptD_{bridge}, we monitored the levels of LptD_{C1–C2}, a folding intermediate with a non-native disulfide bond that accumulates when oxidative folding is impaired (Figures 3C and 3D).¹⁹ It is interesting to note that we observed a significant enhancement in the fitness of *lptD_{bridge} ΔyedD* cells when they were grown in minimal medium (Figure 3E). We attribute this enhancement to the alignment between the reduced LPS transport rate in *lptD_{bridge} ΔyedD* cells and the diminished rate at which cells proliferate in minimal medium. Furthermore, we found that the expression of a YedD variant (YedD_{W70G Q71G R72G}) that does not interact with LptD (Figures 3F and 3G) does not rescue the growth defect of *lptD_{bridge} ΔyedD* cells or reduce the accumulation of LPS in the inner membrane, in contrast to the expression of wild-type YedD (Figures 3B and 3H). Thus, we conclude from

Figure 3. YedD is required for the optimal transit of LPS to the cell surface

(A) The fitness and outer membrane integrity of indicated strains were assessed by efficiency of plating assays (EOPs) on LB-agar supplemented or not with 200 μg/mL vancomycin. In all strains, the chromosomal *lptD* allele was deleted, and the listed *lptD* allele was expressed from a pET23/42 plasmid under the control of a leaky T7 promoter at levels comparable to those produced from the chromosomal *lptD* locus.¹⁸

(B) Immunoblot analysis of whole-cell samples obtained from strains evaluated in (A) and (H) using antibodies directed toward the core region of LPS.

(C) Schematic illustrating the oxidative folding pathway of LptD, leading to its mature, fully oxidized state (LptD_{ox}). The folding of LptD following the formation of the LptD_{C1–C2} intermediate (with a non-native disulfide bond between the first two cysteine residues) represents the rate-limiting step in this pathway.¹⁹ Consequently, disruption of the oxidative folding pathway of LptD leads to the accumulation of LptD_{C1–C2}, with only a small fraction progressing to the mature state.

(D) Immunoblot analysis of whole-cell samples with antibodies directed toward LptD demonstrates that the oxidative folding of LptD_{bridge} proceeds normally. The fast-migrating intermediate LptD_{C1–C2}, observed in the non-reduced sample (without β-mercaptoethanol [β-ME]), does not accumulate when LptD_{bridge} is expressed, similar to what is observed with wild-type LptD. By contrast, the folding-defective variant LptD₄₂₁₃ shows accumulation of this intermediate.⁴⁰ Analyzed samples were obtained from strains evaluated in (A), as well as from a strain carrying the *lptD₄₂₁₃* allele in place of wild-type *lptD* on the chromosome. Note that LptD₄₂₁₃ migrates faster than LptD_{wt} and LptD_{bridge} because it lacks 23 amino acids from an extracellular loop.

(E) The fitness of strains evaluated on LB-agar in (A) was evaluated by EOPs on M9 minimal medium-agar.

(F) View of the interaction interface of YedD with LptD. The LptD C-terminal domain is represented in light blue and the N-terminal domain in dark blue, with the N-terminal extension shown in magenta. The two non-consecutive disulfide bonds present in LptD are shown as orange sticks. Luminal loop 1 and the β strand 1 that follows it are shown in sand, whereas luminal loop 2 and the β strand 26 that precedes it are shown in teal. The C-terminal extension of LptD immediately after loop 2 is depicted in purple. LptE is represented in light pink and YedD in pale cyan. Amino acids in YedD highlighted as green sticks (W70, Q71, and R72) were substituted with glycines for the experiments shown in (B), (G), and (H).

(G) Strep-Tactin affinity purification was performed on outer membrane extracts (input) of a *ΔyedD* strain containing Strep-tagged YedD or YedD_{W70G Q71G R72G} over-expressed from the high copy number pAG776 plasmid. Co-purified partners (pull-down) were separated by SDS-PAGE and detected by immunoblot analysis using antibodies directed toward LptD or Strep-tag II (to detect YedD_{Strep}).

(H) The fitness of the *lptD_{bridge} ΔyedD* strain complemented with pAM238 vectors expressing YedD or the YedD_{W70G Q71G R72G} variant at low levels was assessed by EOPs on LB-agar. The *lptD_{bridge} ΔyedD* strain carrying the empty pAM238 plasmid (pCtrl) was used as a control. Note that the *lptD_{bridge}* variant is present on the pET23/42 plasmid, while the chromosomal *lptD* copy is deleted.

(I) The fitness and outer membrane integrity of indicated strains were assessed by EOPs on LB-agar supplemented or not with 50 or 75 μg/mL vancomycin. For all strains, the chromosomal *lptB* allele was deleted, and the listed *lptB* allele was expressed at low levels from the pET23/42 plasmid, driven by a leaky T7 promoter. The images shown in (A), (B), (D), (E), and (G)–(I) are representative of three independent experiments. See also Figure S5.

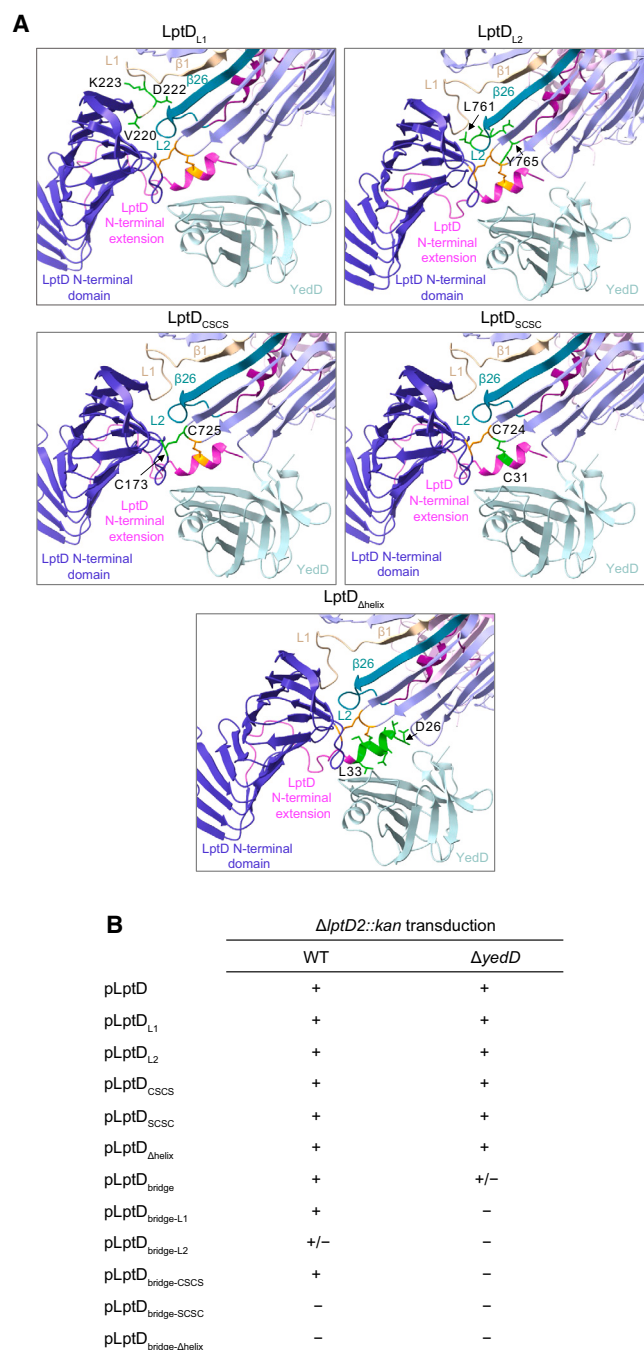


Figure 4. YedD becomes essential for viability when Lpt bridge destabilization is combined with LptDE functional defects

(A) Ribbon diagram representation of the hinge region of LptD mutants used in experiments shown in (B) and of the positioning of YedD relative to these mutations. The LptD C-terminal domain is represented in light blue and the N-terminal domain in dark blue, with the N-terminal extension shown in magenta. The two non-consecutive disulfide bonds present in LptD are shown as orange sticks. Luminal loop 1 (L1) and the β strand 1 (β 1) that follows it are shown in sand, whereas luminal L2 and the β 26 that precedes it are shown in teal. The C-terminal extension of LptD immediately after L2 is depicted in purple. LptE is represented in light pink and YedD in pale cyan. The amino acids shown as green sticks and labeled correspond to the substitutions or

these experiments that the absence of YedD exacerbates the phenotype of cells in which the Lpt bridge is destabilized.

We then tested the impact of altering the hinge region between the β -taco and β -barrel domains of LptD, both in wild-type LptD and in LptD_{bridge}. First, we engineered LptD variants with mutations in L1 and L2 from the luminal gate through which LPS molecules enter the outer membrane. In variants LptD_{L1} and LptD_{bridge-L1}, residues V220, D222, and K223 were replaced with glycines to disrupt the contact with L2; in variants LptD_{L2} and LptD_{bridge-L2}, residues L761 to Y765 were mutated to glycines to prevent the interaction between L2 and the short N-terminal α helix (Figure 4A). We then tested the ability of these variants, which were expressed at physiological levels from a plasmid,¹⁸ to support bacterial growth following transduction of a null *ΔlptD2::kan* allele both in cells expressing *yedD* and in cells lacking *yedD*. We found that the growth of cells expressing LptD_{L1} and LptD_{L2} was not affected (Figures 4B and S6), regardless of the presence or absence of YedD. The situation was strikingly different in cells expressing LptD_{bridge-L1} or LptD_{bridge-L2}: in that case, deletion of *yedD* caused a lethal phenotype (Figures 4B and S6). Next, we introduced specific mutations to disrupt the formation of one of the two disulfide bonds in LptD and LptD_{bridge} (LptD_{CSCS}, LptD_{SCSC}, LptD_{bridge-CSCS}, and LptD_{bridge-SCSC}; Figure 4A) and evaluated the functionality of these variants as described above. While the deletion of *yedD* did not affect cells expressing LptD_{CSCS} or LptD_{SCSC}, it resulted in lethality in cells carrying LptD_{bridge-CSCS} (Figures 4B and S6). We were unable to test cells expressing LptD_{bridge-SCSC} due to the inability of this variant to replace the endogenously expressed LptD (Figures 4B and S6). The C1–C3 disulfide bond anchors the short N-terminal helix of the β -taco domain to the β -barrel. Intriguingly, molecular dynamics simulations have demonstrated the unfolding of this short α helix in the absence of the native disulfide bonds.¹⁶ Consequently, it is plausible that cells expressing LptD_{bridge-SCSC} are nonviable due to the loss of the α helix, thereby hindering the formation of the interaction with YedD. Accordingly, a strain expressing an LptD_{bridge} variant in which the N-terminal α helix has been deleted (residues A25–L33; LptD_{bridge-Δhelix}) is also nonviable (Figures 4A, 4B, and S6). Thus, we conclude that when the LptDE translocon is compromised due to mutations in the LptD hinge region and through alterations in the LptD–LptA interaction, YedD becomes indispensable for cell viability.

deletions present in each LptD variant. In LptD_{L1} variants, residues V220, D222, and K223 were replaced by glycines; in LptD_{L2} variants, residues L761–Y765 were mutated to glycines; in LptD_{CSCS} and LptD_{SCSC} variants, residues C173 and C725 or C31 and C724 were changed to serines, respectively; and in LptD_{Δhelix} variants, the short N-terminal α helix (residues A25–L33) was deleted.

(B) The viability of haploid *lptD* mutants in the presence or absence of YedD was assessed. P1 transduction was used to replace the chromosomal *lptD* copy with a null *ΔlptD2::kan* allele in wild-type (WT) or *ΔyedD* strains expressing the indicated *lptD* allele from a leaky pET23/42 plasmid at levels comparable to those produced from the chromosomal *lptD* locus.¹⁸ + indicates that a large number of normal-sized colonies were obtained following P1 transduction, +/- means a decreased number of and/or small-sized colonies were observed, and – signifies that no colonies grew. The data shown are representative of three independent experiments. Images of the plates obtained following P1 transduction are shown in Figure S6.

Finally, we investigated the impact of combining *yedD* deletion with mutations that impair the function of the Lpt system without affecting the stability of the Lpt bridge. For this purpose, we introduced mutations into LptB, the dimeric ATPase component of the LptB₂FGC complex (Figure 1A).³ Specifically, we selected the E86Q, R150A, and R198A mutations, which were previously shown to reduce the functionality of LptB₂FGC without abolishing it.^{41–43} Our results indicate that deleting *yedD* in cells carrying *lptB*_{E86Q}, *lptB*_{R150A}, or *lptB*_{R198A} as the sole *lptB* copy did not affect their fitness or exacerbate their sensitivity to vancomycin (Figure 3I).

DISCUSSION

The Lpt system is a complex multicomponent nanomachine that ensures the extraction of LPS molecules from the inner membrane, their transport across the hydrophilic periplasm, and their insertion into the outer leaflet of the outer membrane. This intricate machinery, essential in most bacteria, has attracted considerable attention since its discovery in 2005. Initially, the essential components were identified, followed by the elucidation of mechanistic and structural details.³ More recently, single-molecule tracking experiments have shown that the organization of the Lpt bridge is highly dynamic and have proposed a mechanism by which LPS production is directly coupled to its transport.⁴⁴ Additionally, novel antibiotics targeting the Lpt system have been identified.^{45–49} However, crucial questions remain regarding how the action of the different Lpt components is coordinated and controlled, especially at the level of the LptDE translocon. Here, we report the identification of the lipoprotein YedD as a component of the Lpt bridge in *E. coli* and Enterobacterales (see below). We show that YedD specifically interacts with the LptDE translocon, forming stable complexes with a 1:1:1 stoichiometry (Figure 1). Structural analysis of the complex identified the binding interface; YedD interacts with a short N-terminal α helix of the β -taco domain of LptD located in the hinge region between the β -taco and the β -barrel domains (Figure 2). The interaction interface was validated using site-specific photo-crosslinking; interactions with LptE were also detected, likely through contacts with the flexible C terminus of this lipoprotein (Figures 2 and S5).

LptD, a protein known for its complex folding process, has been shown to interact with two proteins other than LptE: BepA, a periplasmic protein with both chaperone and protease activities, and the lipoprotein YifL.^{50,51} Both BepA and YifL have been implicated in the maturation of LptD but do not participate in Lpt activity. YedD, however, markedly differs from BepA and YifL. Indeed, our results show that YedD is crucial for the normal functioning of Lpt: the absence of YedD impairs cellular viability and leads to LPS accumulation in the inner membrane under conditions where the Lpt bridge is compromised (Figures 3 and 4). Although YedD is not essential under laboratory conditions, and its absence does not affect LPS transport when other components of the Lpt bridge function properly, its absence becomes detrimental when mutations destabilize the interface between the LptDE translocon and LptA. This observation suggests that YedD contributes to stabilizing the Lpt bridge in an active conformation. Furthermore, the fact that *yedD* deletion

does not exacerbate the phenotype of cells with reduced Lpt function due to LptB mutations—rather than decreased bridge stability—provides additional support for the idea that YedD specifically stabilizes the Lpt bridge. Interestingly, a recent report proposes that two types of bridges may exist, differing in stability based on the presence (long lived) or absence (short lived) of LPS molecules.⁴⁴ It is, therefore, possible that YedD contributes to bridge stability when LPS is present. To test for direct interactions between YedD and LPS molecules, we introduced pBpa at 71 different sites throughout the entire 3D structure of YedD and searched for YedD-LPS crosslinks. However, we did not detect any crosslinks, suggesting that YedD does not directly bind LPS (data not shown). Our data further indicate that YedD is also necessary for fine-tuning the LptDE translocon, particularly under conditions of high LPS flux (Figure 3). Indeed, we found that while cells tolerate mutations affecting LptDE activity if YedD is present (Figure 4), they become severely impaired when YedD is absent. YedD might, for instance, be necessary for ensuring the correct positioning of the β -taco domain of LptD relative to the β -barrel domain, which is crucial for the exit of LPS through the β -barrel lateral gate and priming of the LptDE-LptA interaction.^{11,24,52} As bridge stability and translocon activity are likely interdependent, YedD appears to function as an Lpt optimizer. Additional research into the mechanistic details of how LptDE facilitates the insertion of LPS molecules into the outer leaflet of the outer membrane is essential to gain further insight into how YedD impacts LPS transport.

YedD is highly conserved within the Enterobacterales order (Figure S7 and Data S1). It is present in numerous human pathogens, including *Enterobacter* spp. and *Klebsiella pneumoniae* strains from the ESKAPE group of highly virulent and antibiotic-resistant bacteria.⁵³ Maintaining an optimal outer membrane barrier in the hostile host environment is critical for the survival of pathogens, and the role of YedD may, therefore, be crucial for virulence. Interestingly, the size and structural features of the C-terminal β -barrel domain of LptD homologs are highly conserved, but their N-terminal domains are more variable.¹⁷ For example, the periplasmic β -taco domain of *Pseudomonas aeruginosa* LptD includes a long N-terminal insert of approximately 100 residues.^{17,54} This raises the question of whether YedD functional analogs, tailored to adapt to the structure of the LptD periplasmic domain, exist in organisms that do not possess YedD or if alternative regulatory mechanisms of LPS translocation to the surface occur.

YedD belongs to the widespread lipocalin family. Despite the high conservation of the structural features of lipocalins, comparisons of their primary sequences reveal unusually low identity among them, complicating the identification of new lipocalin family members without structural data.²⁸ According to phylogenetic analyses and available structures, lipocalins have not yet been identified in prokaryotes that lack an outer membrane,^{55–57} leading to the hypothesis that bacterial lipocalins may be involved in envelope biogenesis. Our work provides the first clear example of such a protein unequivocally involved in this process. Therefore, it is plausible that Blc,⁵⁵ another lipocalin expressed in the *E. coli* envelope, may also play a role in optimizing the function of essential envelope building and maintenance pathways.

Due to its crucial role in outer membrane biogenesis, the LptDE translocon has emerged as a key target in antibiotic discovery. Recently, an antibiotic that traps LPS within the Lpt bridge of *Acinetobacter baumannii* was identified.^{47,58} Additionally, a family of synthetic peptidomimetic antibiotics that specifically inhibit LPS transport to the cell surface by targeting *P. aeruginosa* LptD has been discovered.^{46,59} One closely related molecule, murepavadin, is currently in the early stages of clinical development for use as a *Pseudomonas*-specific antibiotic in patients with critical lung infections.⁶⁰ Interestingly, this family of peptidomimetics targets the periplasmic domain of LptD, with one binding site located in the N-terminal insert domain.^{46,54} The discovery of antimicrobial molecules that target the β -taco domain of LptD underscores the pivotal role of this hub region in LPS transport across the envelope to its final destination. The interaction of YedD with this domain introduces an additional layer of complexity that must be considered in the future design of antibacterial drugs targeting the Lpt machinery.

Limitations of the study

While this study provides significant insights into the role of YedD in stabilizing the Lpt bridge and facilitating LPS transport, some limitations should be acknowledged. First, although we establish *yedD* as a bona fide component of the Lpt system and provide evidence indicating its role in stabilizing the bridge, its precise molecular function remains unclear. Second, our experiments were performed under laboratory conditions, which may not fully replicate the physiological stressors or environmental contexts in which YedD operates in *E. coli*. These limitations underscore the need for further studies to comprehensively define YedD's role in Lpt system activity and outer membrane biogenesis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jean-François Collet (jfcollet@uclouvain.be).

Materials availability

Strains and plasmids generated in this study are available upon request to the lead contact.

Data and code availability

- Coordinates and the electron potential maps for the LptDE-YedD cryo-EM structure have been deposited in PDB and EMDB under accession codes PDB: 9FZ5 and EMBL-EBI: EMD-50893, respectively, and are publicly available as of the date of publication. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PRIDE: PXD059551 and are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.G., H.R., and J.-F.C.; investigation, strain construction, construct cloning, protein expression, and purification, A.G., N.C., and L.T.; writing, J.-F.C., H.R., and A.G.; proteomics, D.V.; structure determination and analysis, V.S.N. and H.R.; interactive molecular dynamics flexible fitting of the cryo-EM model and phylogenetic analysis, B.I.I.; data analysis and interpretation, A.G., V.S.N., H.R., N.C., L.T., B.I.I., and J.-F.C. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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REFERENCES

- Silhavy, T.J., Kahne, D., and Walker, S. (2010). The bacterial cell envelope. Cold Spring Harbor Perspect. Biol. 2, a000414. <https://doi.org/10.1101/cshperspect.a000414>.

2. Guest, R.L., and Silhavy, T.J. (2023). Cracking outer membrane biogenesis. *Biochim. Biophys. Acta. Mol. Cell Res.* 1870, 119405. <https://doi.org/10.1016/j.bbamcr.2022.119405>.
3. Lundstedt, E., Kahne, D., and Ruiz, N. (2021). Assembly and Maintenance of Lipids at the Bacterial Outer Membrane. *Chem. Rev.* 121, 5098–5123. <https://doi.org/10.1021/acs.chemrev.0c00587>.
4. Bertani, B., and Ruiz, N. (2018). Function and Biogenesis of Lipopolysaccharides. *EcoSal Plus* 8, 1–19. <https://doi.org/10.1128/ecosalplus.ESP-0001-2018>.
5. Rojas, E.R., Billings, G., Odermatt, P.D., Auer, G.K., Zhu, L., Miguel, A., Chang, F., Weibel, D.B., Theriot, J.A., and Huang, K.C. (2018). The outer membrane is an essential load-bearing element in Gram-negative bacteria. *Nature* 559, 617–621. <https://doi.org/10.1038/s41586-018-0344-3>.
6. Deghelt, M., Cho, S.-H., Govers, S.K., Janssens, A., Dachsbeck, A., Remaut, H.K., and Collet, J.-F. (2023). The outer membrane and peptidoglycan layer form a single mechanical device balancing turgor. Preprint at bioRxiv. <https://doi.org/10.1101/2023.04.29.538579>.
7. El Rayes, J., Rodríguez-Alonso, R., and Collet, J.F. (2021). Lipoproteins in Gram-negative bacteria: new insights into their biogenesis, subcellular targeting and functional roles. *Curr. Opin. Microbiol.* 61, 25–34. <https://doi.org/10.1016/j.mib.2021.02.003>.
8. Troman, L.A., and Collinson, I. (2021). Pushing the Envelope: The Mysterious Journey Through the Bacterial Secretory Machinery, and Beyond. *Front. Microbiol.* 12, 782900. <https://doi.org/10.3389/fmicb.2021.782900>.
9. Tomasek, D., and Kahne, D. (2021). The assembly of beta-barrel outer membrane proteins. *Curr. Opin. Microbiol.* 60, 16–23. <https://doi.org/10.1016/j.mib.2021.01.009>.
10. Suits, M.D.L., Sperandeo, P., Dehò, G., Polissi, A., and Jia, Z. (2008). Novel structure of the conserved gram-negative lipopolysaccharide transport protein A and mutagenesis analysis. *J. Mol. Biol.* 380, 476–488. <https://doi.org/10.1016/j.jmb.2008.04.045>.
11. Freinkman, E., Okuda, S., Ruiz, N., and Kahne, D. (2012). Regulated assembly of the transenvelope protein complex required for lipopolysaccharide export. *Biochemistry* 51, 4800–4806. <https://doi.org/10.1021/bi300592c>.
12. Owens, T.W., Taylor, R.J., Pahil, K.S., Bertani, B.R., Ruiz, N., Kruse, A.C., and Kahne, D. (2019). Structural basis of unidirectional export of lipopolysaccharide to the cell surface. *Nature* 567, 550–553. <https://doi.org/10.1038/s41586-019-1039-0>.
13. Qiao, S., Luo, Q., Zhao, Y., Zhang, X.C., and Huang, Y. (2014). Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* 511, 108–111. <https://doi.org/10.1038/nature13484>.
14. Okuda, S., Freinkman, E., and Kahne, D. (2012). Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science* 338, 1214–1217. <https://doi.org/10.1126/science.1228984>.
15. Sherman, D.J., Xie, R., Taylor, R.J., George, A.H., Okuda, S., Foster, P.J., Needleman, D.J., and Kahne, D. (2018). Lipopolysaccharide is transported to the cell surface by a membrane-to-membrane protein bridge. *Science* 359, 798–801. <https://doi.org/10.1126/science.aar1886>.
16. Gu, Y., Stansfeld, P.J., Zeng, Y., Dong, H., Wang, W., and Dong, C. (2015). Lipopolysaccharide is inserted into the outer membrane through an intramembrane hole, a lumen gate, and the lateral opening of LptD. *Structure* 23, 496–504. <https://doi.org/10.1016/j.str.2015.01.001>.
17. Botos, I., Majdalani, N., Mayclin, S.J., McCarthy, J.G., Lundquist, K., Wojtowicz, D., Barnard, T.J., Gumbart, J.C., and Buchanan, S.K. (2016). Structural and Functional Characterization of the LPS Transporter LptDE from Gram-Negative Pathogens. *Structure* 24, 965–976. <https://doi.org/10.1016/j.str.2016.03.026>.
18. Ruiz, N., Chng, S.S., Hiniker, A., Kahne, D., and Silhavy, T.J. (2010). Nonconsecutive disulfide bond formation in an essential integral outer membrane protein. *Proc. Natl. Acad. Sci. USA* 107, 12245–12250. <https://doi.org/10.1073/pnas.1007319107>.
19. Chng, S.S., Xue, M., Garner, R.A., Kadokura, H., Boyd, D., Beckwith, J., and Kahne, D. (2012). Disulfide rearrangement triggered by translocon assembly controls lipopolysaccharide export. *Science* 337, 1665–1668. <https://doi.org/10.1126/science.1227215>.
20. Freinkman, E., Chng, S.S., and Kahne, D. (2011). The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc. Natl. Acad. Sci. USA* 108, 2486–2491. <https://doi.org/10.1073/pnas.1015617108>.
21. Dong, H., Xiang, Q., Gu, Y., Wang, Z., Paterson, N.G., Stansfeld, P.J., He, C., Zhang, Y., Wang, W., and Dong, C. (2014). Structural basis for outer membrane lipopolysaccharide insertion. *Nature* 511, 52–56. <https://doi.org/10.1038/nature13464>.
22. Malojcic, G., Andres, D., Grabowicz, M., George, A.H., Ruiz, N., Silhavy, T.J., and Kahne, D. (2014). LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. *Proc. Natl. Acad. Sci. USA* 111, 9467–9472. <https://doi.org/10.1073/pnas.1402746111>.
23. Li, X., Gu, Y., Dong, H., Wang, W., and Dong, C. (2015). Trapped lipopolysaccharide and LptD intermediates reveal lipopolysaccharide translocation steps across the *Escherichia coli* outer membrane. *Sci. Rep.* 5, 11883. <https://doi.org/10.1038/srep11883>.
24. Lundquist, K.P., and Gumbart, J.C. (2020). Presence of substrate aids lateral gate separation in LptD. *Biochim. Biophys. Acta. Biomembr.* 1862, 183025. <https://doi.org/10.1016/j.bbamem.2019.07.013>.
25. Botte, M., Ni, D., Schenck, S., Zimmermann, I., Chami, M., Bocquet, N., Egloff, P., Bucher, D., Trabuco, M., Cheng, R.K.Y., et al. (2022). Cryo-EM structures of a LptDE transporter in complex with Pro-macrobodies offer insight into lipopolysaccharide translocation. *Nat. Commun.* 13, 1826. <https://doi.org/10.1038/s41467-022-29459-2>.
26. Xie, R., Taylor, R.J., and Kahne, D. (2018). Outer Membrane Translocon Communicates with Inner Membrane ATPase To Stop Lipopolysaccharide Transport. *J. Am. Chem. Soc.* 140, 12691–12694. <https://doi.org/10.1021/jacs.8b07656>.
27. Villa, R., Martorana, A.M., Okuda, S., Gourlay, L.J., Nardini, M., Sperandeo, P., Dehò, G., Bolognesi, M., Kahne, D., and Polissi, A. (2013). The *Escherichia coli* Lpt transenvelope protein complex for lipopolysaccharide export is assembled via conserved structurally homologous domains. *J. Bacteriol.* 195, 1100–1108. <https://doi.org/10.1128/JB.02057-12>.
28. Flower, D.R., North, A.C., and Sansom, C.E. (2000). The lipocalin protein family: structural and sequence overview. *Biochim. Biophys. Acta* 1482, 9–24. [https://doi.org/10.1016/S0167-4838\(00\)00148-5](https://doi.org/10.1016/S0167-4838(00)00148-5).
29. Schiefner, A., and Skerra, A. (2015). The menagerie of human lipocalins: a natural protein scaffold for molecular recognition of physiological compounds. *Acc. Chem. Res.* 48, 976–985. <https://doi.org/10.1021/ar5003973>.
30. Chng, S.S., Gronenberg, L.S., and Kahne, D. (2010). Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry* 49, 4565–4567. <https://doi.org/10.1021/bi100493e>.
31. Redl, B., and Habeler, M. (2022). The diversity of lipocalin receptors. *Biochimie* 192, 22–29. <https://doi.org/10.1016/j.biochi.2021.09.008>.
32. Zanotti, G., and Berni, R. (2004). Plasma retinol-binding protein: structure and interactions with retinol, retinoids, and transthyretin. *Vitam. Horm.* 69, 271–295. [https://doi.org/10.1016/S0083-6729\(04\)69010-8](https://doi.org/10.1016/S0083-6729(04)69010-8).
33. Borregaard, N., and Cowland, J.B. (2006). Neutrophil gelatinase-associated lipocalin, a siderophore-binding eukaryotic protein. *Biometals* 19, 211–215. <https://doi.org/10.1007/s10534-005-3251-7>.
34. Schiefner, A., Rodewald, F., Neumaier, I., and Skerra, A. (2015). The dimeric crystal structure of the human fertility lipocalin glycodefin reveals a protein scaffold for the presentation of complex glycans. *Biochem. J.* 466, 95–104. <https://doi.org/10.1042/BJ20141003>.
35. Young, T.S., Ahmad, I., Yin, J.A., and Schultz, P.G. (2010). An enhanced system for unnatural amino acid mutagenesis in *E. coli*. *J. Mol. Biol.* 395, 361–374. <https://doi.org/10.1016/j.jmb.2009.10.030>.
36. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction

- of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2, 2006.0008. <https://doi.org/10.1038/msb4100050>.
37. Sutterlin, H.A., Zhang, S., and Silhavy, T.J. (2014). Accumulation of phosphatidic acid increases vancomycin resistance in *Escherichia coli*. *J. Bacteriol.* 196, 3214–3220. <https://doi.org/10.1128/JB.01876-14>.
38. Sperandio, P., Lau, F.K., Carpentieri, A., De Castro, C., Molinaro, A., Dehò, G., Silhavy, T.J., and Polissi, A. (2008). Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J. Bacteriol.* 190, 4460–4469. <https://doi.org/10.1128/JB.00270-08>.
39. Ruiz, N., Gronenberg, L.S., Kahne, D., and Silhavy, T.J. (2008). Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 105, 5537–5542. <https://doi.org/10.1073/pnas.0801196105>.
40. Lee, J., Xue, M., Wzorek, J.S., Wu, T., Grabowicz, M., Gronenberg, L.S., Sutterlin, H.A., Davis, R.M., Ruiz, N., Silhavy, T.J., and Kahne, D.E. (2016). Characterization of a stalled complex on the beta-barrel assembly machine. *Proc. Natl. Acad. Sci. USA* 113, 8717–8722. <https://doi.org/10.1073/pnas.1604100113>.
41. Lundstedt, E.A., Simpson, B.W., and Ruiz, N. (2020). LptB-LptF coupling mediates the closure of the substrate-binding cavity in the LptB(2) FGC transporter through a rigid-body mechanism to extract LPS. *Mol. Microbiol.* 114, 200–213. <https://doi.org/10.1111/mmi.14506>.
42. Simpson, B.W., Owens, T.W., Orabella, M.J., Davis, R.M., May, J.M., Trauger, S.A., Kahne, D., and Ruiz, N. (2016). Identification of Residues in the Lipopolysaccharide ABC Transporter That Coordinate ATPase Activity with Extrator Function. *mBio* 7, e01729-16. <https://doi.org/10.1128/mBio.01729-16>.
43. Simpson, B.W., Pahil, K.S., Owens, T.W., Lundstedt, E.A., Davis, R.M., Kahne, D., and Ruiz, N. (2019). Combining Mutations That Inhibit Two Distinct Steps of the ATP Hydrolysis Cycle Restores Wild-Type Function in the Lipopolysaccharide Transporter and Shows that ATP Binding Triggers Transport. *mBio* 10, e01931-19. <https://doi.org/10.1128/mBio.01931-19>.
44. Tork, L., Moffatt, C.B., Bernhardt, T.G., Garner, E.C., and Kahne, D. (2023). Single-molecule dynamics show a transient lipopolysaccharide transport bridge. *Nature* 623, 814–819. <https://doi.org/10.1038/s41586-023-06709-x>.
45. Shankaramma, S.C., Athanassiou, Z., Zerbe, O., Moehle, K., Mouton, C., Bernardini, F., Vrijbloed, J.W., Obrecht, D., and Robinson, J.A. (2002). Macrocyclic hairpin mimetics of the cationic antimicrobial peptide protegrin I: a new family of broad-spectrum antibiotics. *ChemBiochem* 3, 1126–1133. [https://doi.org/10.1002/1439-7633\(20021104\)3:11<1126::AID-CBIC1126>3.0.CO;2-I](https://doi.org/10.1002/1439-7633(20021104)3:11<1126::AID-CBIC1126>3.0.CO;2-I).
46. Srinivas, N., Jetter, P., Ueberbacher, B.J., Werneburg, M., Zerbe, K., Steinmann, J., Van der Meijden, B., Bernardini, F., Lederer, A., Dias, R.L.A., et al. (2010). Peptidomimetic antibiotics target outer-membrane biogenesis in *Pseudomonas aeruginosa*. *Science* 327, 1010–1013. <https://doi.org/10.1126/science.1182749>.
47. Pahil, K.S., Gilman, M.S.A., Baidin, V., Clairfeuille, T., Mattei, P., Bieniossek, C., Dey, F., Muri, D., Baettig, R., Lobritz, M., et al. (2024). A new antibiotic traps lipopolysaccharide in its intermembrane transporter. *Nature* 625, 572–577. <https://doi.org/10.1038/s41586-023-06799-7>.
48. Vetterli, S.U., Zerbe, K., Müller, M., Urfer, M., Mondal, M., Wang, S.Y., Moehle, K., Zerbe, O., Vitale, A., Pessi, G., et al. (2018). Thanatin targets the intermembrane protein complex required for lipopolysaccharide transport in *Escherichia coli*. *Sci. Adv.* 4, eaau2634. <https://doi.org/10.1126/sciadv.aau2634>.
49. Schuster, M., Brabet, E., Oi, K.K., Desjonquères, N., Moehle, K., Le Poupon, K., Hell, S., Gable, S., Rithié, V., Dillinger, S., et al. (2023). Peptidomimetic antibiotics disrupt the lipopolysaccharide transport bridge of drug-resistant *Enterobacteriaceae*. *Sci. Adv.* 9, eadg3683. <https://doi.org/10.1126/sciadv.adg3683>.
50. Miyazaki, R., Watanabe, T., Yoshitani, K., and Akiyama, Y. (2021). Edge-strand of BepA interacts with immature LptD on the beta-barrel assembly machine to direct it to on- and off-pathways. *Elife* 10, e70541. <https://doi.org/10.7554/eLife.70541>.
51. Yang, Y., Chen, H., Corey, R.A., Morales, V., Quentin, Y., Froment, C., Caumont-Sarcos, A., Albenne, C., Burlet-Schiltz, O., Ranava, D., et al. (2023). LptM promotes oxidative maturation of the lipopolysaccharide translocator by substrate binding mimicry. *Nat. Commun.* 14, 6368. <https://doi.org/10.1038/s41467-023-42007-w>.
52. Fiorentino, F., Sauer, J.B., Qiu, X., Corey, R.A., Cassidy, C.K., Mynors-Wallis, B., Mehmood, S., Bolla, J.R., Stansfeld, P.J., and Robinson, C.V. (2021). Dynamics of an LPS translocator induced by substrate and an antimicrobial peptide. *Nat. Chem. Biol.* 17, 187–195. <https://doi.org/10.1038/s41589-020-00694-2>.
53. Miller, W.R., and Arias, C.A. (2024). ESKAPE pathogens: antimicrobial resistance, epidemiology, clinical impact and therapeutics. *Nat. Rev. Microbiol.* 22, 598–616. <https://doi.org/10.1038/s41579-024-01054-w>.
54. Andolina, G., Bencze, L.C., Zerbe, K., Müller, M., Steinmann, J., Kocherla, H., Mondal, M., Sobek, J., Moehle, K., Malojčić, G., et al. (2018). A Peptidomimetic Antibiotic Interacts with the Periplasmic Domain of LptD from *Pseudomonas aeruginosa*. *ACS Chem. Biol.* 13, 666–675. <https://doi.org/10.1021/acscchembio.7b00822>.
55. Bishop, R.E. (2000). The bacterial lipocalins. *Biochim. Biophys. Acta* 1482, 73–83. [https://doi.org/10.1016/S0167-4838\(00\)00138-2](https://doi.org/10.1016/S0167-4838(00)00138-2).
56. Ganfornina, M.D., Gutiérrez, G., Bastiani, M., and Sánchez, D. (2000). A phylogenetic analysis of the lipocalin protein family. *Mol. Biol. Evol.* 17, 114–126. <https://doi.org/10.1093/oxfordjournals.molbev.a026224>.
57. Gutierrez, G., Ganfornina, M.D., and Sanchez, D. (2000). Evolution of the lipocalin family as inferred from a protein sequence phylogeny. *Biochim. Biophys. Acta* 1482, 35–45. [https://doi.org/10.1016/S0167-4838\(00\)00151-5](https://doi.org/10.1016/S0167-4838(00)00151-5).
58. Zampaloni, C., Mattei, P., Bleicher, K., Winther, L., Thäte, C., Bucher, C., Adam, J.M., Alanine, A., Amrein, K.E., Baidin, V., et al. (2024). A novel antibiotic class targeting the lipopolysaccharide transporter. *Nature* 625, 566–571. <https://doi.org/10.1038/s41586-023-06873-0>.
59. Werneburg, M., Zerbe, K., Juhas, M., Bigler, L., Stalder, U., Kaeche, A., Ziegler, U., Obrecht, D., Eberl, L., and Robinson, J.A. (2012). Inhibition of lipopolysaccharide transport to the outer membrane in *Pseudomonas aeruginosa* by peptidomimetic antibiotics. *ChemBiochem* 13, 1767–1775. <https://doi.org/10.1002/cbic.201200276>.
60. Spexis (2024). Inhaled Murepavadin. <https://spexisbio.com/impv/>.
61. Guyer, M.S., Reed, R.R., Steitz, J.A., and Low, K.B. (1981). Identification of a sex-factor-affinity site in *E. coli* as gamma delta. *Cold Spring. Harb. Symp. Quant. Biol.* 45, 135–140. <https://doi.org/10.1101/sqb.1981.045.01.022>.
62. Casadaban, M.J. (1976). Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* 104, 541–555. [https://doi.org/10.1016/0022-2836\(76\)90119-4](https://doi.org/10.1016/0022-2836(76)90119-4).
63. Majdalani, N., Hernandez, D., and Gottesman, S. (2002). Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol. Microbiol.* 46, 813–826. <https://doi.org/10.1046/j.1365-2958.2002.03203.x>.
64. Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97, 6640–6645. <https://doi.org/10.1073/pnas.120163297>.
65. Miroux, B., and Walker, J.E. (1996). Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260, 289–298. <https://doi.org/10.1006/jmbi.1996.0399>.
66. Chng, S.S., Ruiz, N., Chimalakonda, G., Silhavy, T.J., and Kahne, D. (2010). Characterization of the two-protein complex in *Escherichia coli*

- responsible for lipopolysaccharide assembly at the outer membrane. *Proc. Natl. Acad. Sci. USA* 107, 5363–5368. <https://doi.org/10.1073/pnas.0912872107>.
67. Dekoninck, K., Letoquart, J., Laguri, C., Demange, P., Bevernaegie, R., Simorre, J.P., Dehu, O., Iorga, B.I., Elias, B., Cho, S.H., and Collet, J.F. (2020). Defining the function of OmpA in the Rcs stress response. *Elife* 9, e60861. <https://doi.org/10.7554/eLife.60861>.
 68. Datta, S., Costantino, N., and Court, D.L. (2006). A set of recombinering plasmids for gram-negative bacteria. *Gene* 379, 109–115. <https://doi.org/10.1016/j.gene.2006.04.018>.
 69. Cherepanov, P.P., and Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158, 9–14. [https://doi.org/10.1016/0378-1119\(95\)00193-a](https://doi.org/10.1016/0378-1119(95)00193-a).
 70. Wu, T., McCandlish, A.C., Gronenberg, L.S., Chng, S.S., Silhavy, T.J., and Kahne, D. (2006). Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 103, 11754–11759. <https://doi.org/10.1073/pnas.0604744103>.
 71. Gil, D., and Bouché, J.P. (1991). ColE1-type vectors with fully repressible replication. *Gene* 105, 17–22. [https://doi.org/10.1016/0378-1119\(91\)90508-9](https://doi.org/10.1016/0378-1119(91)90508-9).
 72. Amann, E., Ochs, B., and Abel, K.J. (1988). Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* 69, 301–315. [https://doi.org/10.1016/0378-1119\(88\)90440-4](https://doi.org/10.1016/0378-1119(88)90440-4).
 73. Mastronarde, D.N. (2005). Automated electron microscope tomography using robust prediction of specimen movements. *J. Struct. Biol.* 152, 36–51. <https://doi.org/10.1016/j.jmb.2005.07.007>.
 74. Zheng, S.Q., Palovcak, E., Armache, J.P., Verba, K.A., Cheng, Y., and Agard, D.A. (2017). MotionCorr2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14, 331–332. <https://doi.org/10.1038/nmeth.4193>.
 75. Rohou, A., and Grigorieff, N. (2015). CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* 192, 216–221. <https://doi.org/10.1016/j.jmb.2015.08.008>.
 76. Wagner, T., Merino, F., Stabrin, M., Moriya, T., Antoni, C., Apelbaum, A., Hagel, P., Sitsel, O., Risch, T., Prumbaum, D., et al. (2019). SPHIRE-crYOLO is a fast and accurate fully automated particle picker for cryo-EM. *Commun. Biol.* 2, 218. <https://doi.org/10.1038/s42003-019-0437-z>.
 77. Zivanov, J., Nakane, T., Forsberg, B.O., Kimanius, D., Hagen, W.J., Lindahl, E., and Scheres, S.H. (2018). New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife* 7, e42166. <https://doi.org/10.7554/eLife.42166>.
 78. Punjani, A., Rubinstein, J.L., Fleet, D.J., and Brubaker, M.A. (2017). cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods* 14, 290–296. <https://doi.org/10.1038/nmeth.4169>.
 79. Kimanius, D., Jamali, K., Wilkinson, M.E., Lövestam, S., Velazhahan, V., Nakane, T., and Scheres, S.H. (2023). Data-driven regularisation lowers the size barrier of cryo-EM structure determination. Preprint at bioRxiv. <https://doi.org/10.1101/2023.10.23.563586>.
 80. He, J., Li, T., and Huang, S.Y. (2023). Improvement of cryo-EM maps by simultaneous local and non-local deep learning. *Nat. Commun.* 14, 3217. <https://doi.org/10.1038/s41467-023-39031-1>.
 81. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Židek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. <https://doi.org/10.1038/s41586-021-03819-2>.
 82. Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612. <https://doi.org/10.1002/jcc.20084>.
 83. Meng, E.C., Goddard, T.D., Pettersen, E.F., Couch, G.S., Pearson, Z.J., Morris, J.H., and Ferrin, T.E. (2023). UCSF ChimeraX: Tools for structure building and analysis. *Protein Sci.* 32, e4792. <https://doi.org/10.1002/pro.4792>.
 84. Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132. <https://doi.org/10.1107/S0907444904019158>.
 85. Afonine, P.V., Poon, B.K., Read, R.J., Sobolev, O.V., Terwilliger, T.C., Urzhumtsev, A., and Adams, P.D. (2018). Real-space refinement in PHENIX for cryo-EM and crystallography. *Acta Crystallogr. D Struct. Biol.* 74, 531–544. <https://doi.org/10.1107/S2059798318006551>.
 86. Chen, V.B., Arendall, W.B., 3rd, Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2010). MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 66, 12–21. <https://doi.org/10.1107/S0907444909042073>.
 87. Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539. <https://doi.org/10.1038/msb.2011.75>.
 88. Jo, S., Kim, T., Iyer, V.G., and Im, W. (2008). CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* 29, 1859–1865. <https://doi.org/10.1002/jcc.20945>.
 89. Phillips, J.C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R.D., Kalé, L., and Schulten, K. (2005). Scalable molecular dynamics with NAMD. *J. Comput. Chem.* 26, 1781–1802. <https://doi.org/10.1002/jcc.20289>.
 90. Demichev, V., Messner, C.B., Vernardis, S.I., Lilley, K.S., and Ralser, M. (2020). DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nat. Methods* 17, 41–44. <https://doi.org/10.1038/s41592-019-0638-x>.
 91. Bremer, E., Silhavy, T.J., Weisemann, J.M., and Weinstock, G.M. (1984). Lambda placMu: a transposable derivative of bacteriophage lambda for creating lacZ protein fusions in a single step. *J. Bacteriol.* 158, 1084–1093. <https://doi.org/10.1128/jb.158.3.1084-1093.1984>.
 92. Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 97, 5978–5983. <https://doi.org/10.1073/pnas.100127597>.
 93. Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. (2014). Recombineering: genetic engineering in bacteria using homologous recombination. *Curr. Protoc. Mol. Biol.* 106, 1.16.1–1.16.39. <https://doi.org/10.1002/0471142727.mb0116s106>.
 94. Gennaris, A., Ezraty, B., Henry, C., Agrebi, R., Vergnes, A., Oheix, E., Bos, J., Leverrier, P., Espinosa, L., Szewczyk, J., et al. (2015). Repairing oxidized proteins in the bacterial envelope using respiratory chain electrons. *Nature* 528, 409–412. <https://doi.org/10.1038/nature15764>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-YedD	CER Group, Marloie, Belgium	N/A
Rabbit polyclonal anti-LptC	Alessandra Polissi, University of Milano, Italy	N/A
Rabbit polyclonal anti-LptD	Shin-Ichi Matsuyama, Rikkyo University, Japan	N/A
Rabbit polyclonal rabbit anti-LptE	Daniel Kahne, Harvard University, USA	N/A
Mouse monoclonal anti-Lipopolysaccharide Core	Hycult Biotech	mAb WN1 222-5; Cat#HM6011
Mouse monoclonal anti-Strep-tag II	IBA Lifesciences	StrepMAB-Classic; Cat#2-1507-001
Anti-rabbit IgG HRP-linked antibody	Cytiva	Cat#NA934
Anti-mouse IgG HRP-linked antibody	Sigma-Aldrich	Cat#A4416
Bacterial and virus strains		
<i>Escherichia coli</i> K12 MG1655	Guyer et al. ⁶¹	CSGC#6300
<i>Escherichia coli</i> K12 MC4100	Casadaban et al. ⁶²	CSGC#6152
<i>Escherichia coli</i> K12 DH300	Majdalani et al. ⁶³	N/A
<i>Escherichia coli</i> K12 BW25113	Datsenko et al. ⁶⁴	CSGC#7636
C43 (DE3)	Miroux et al. ⁶⁵	N/A
Shuffle T7 express	New England Biolabs	Cat#C3029
For a detailed list of all bacterial strains used in this paper, see Table S2	This paper; Baba et al. ³⁶ , Chng et al. ⁶⁶ , Dekoninck et al. ⁶⁷ , Ruiz et al. ³⁹ ; Sutterlin et al. ³⁷	N/A
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Sigma-Aldrich	Cat#A9518
Chloramphenicol	Sigma-Aldrich	Cat#C0378
Kanamycin sulfate	Sigma-Aldrich	Cat#K4000
Spectinomycin dihydrochloride pentahydrate	Sigma-Aldrich	Cat#S4014
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	Cat#11873580001
N-Lauroylsarcosine sodium salt	Sigma-Aldrich	Cat#L5777
<i>n</i> -dodecyl-β-D-maltopyranoside (DDM)	Inalco Pharmaceuticals; Anatrace	Cat#1758-1350; Cat#D310S
Strep-Tactin Sepharose 50% suspension	IBA Lifesciences	Cat#2-1201-025
Strep-Tactin superflow high capacity cartridge, 5 mL	IBA Lifesciences	Cat#2-1240-001
D-Desthiobiotin	Millipore	Cat#71610-3
PageBlue Protein Staining Solution	Thermo Fisher Scientific	Cat#24620
HisPur™ Ni-NTA resin	Thermo Fisher Scientific	Cat#88222
HisTrap HP column, 1 mL	Cytiva	Cat#17524701
HiLoad 16/60 Superdex 75 prep grade column	Cytiva	Cat#17-1068-01
Imidazole	Millipore	Cat#104716
Isopropyl β-D-1-thiogalactopyranoside (IPTG), Dioxane-free	Promega	Cat#V3953
L-(+)-Arabinose	Sigma-Aldrich	Cat#A3256
Acclaim PepMap 100 trap cartridge	Thermo Fisher Scientific	Cat#160434
PepMap RSLC, 0.075 x 250 mm	Thermo Fisher Scientific	Cat#ES902
Sequencing Grade Modified Trypsin	Promega	Cat#V5111
p-benzoyl-L-phenylalanine (pBpa)	Bachem	Cat#F-2800.0001

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
M9 minimal medium salts	MP Biomedicals	Cat#3037012
Vancomycin hydrochloride, Molecular Biology Grade	Alfa Aesar	Cat#J62790
β-Mercaptoethanol	Sigma-Aldrich	Cat#M6250
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat#32106
Immobilon Western Chemiluminescent HRP Substrate	Millipore	Cat#WBKLS0500
NHS-activated Sepharose 4 Fast Flow resin	Cytiva	Cat#17090601

Deposited data

LptDE-YedD Cryo-EM structure coordinates and electron potential maps	This paper	PDB: 9FZ5; EMD: EMD-50893
LptDE structure from <i>Klebsiella pneumoniae</i>	Botos et al. ¹⁷	PDB: 5IV9
YedD structure from <i>Klebsiella pneumoniae</i>	Joint Center for Structural Genomics (JCSG)	PDB: 4HWM
Mass spectrometry proteomics data	This paper	PXD059551

Oligonucleotides

For a detailed list of the PCR primers used in this paper, see Table S4	Integrated DNA Technologies	N/A
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Recombinant DNA

For a detailed list of the plasmids used in this paper, see Table S3	This paper; Novagen; Chng et al. ⁶⁶ ; Dekoninck et al. ⁶⁷ ; Sutterlin et al. ³⁷ ; Datta et al. ⁶⁸ ; Cherepanov et al. ⁶⁹ ; Wu et al. ⁷⁰ ; Gil et al. ⁷¹ ; Young et al. ³⁵ ; Amann et al. ⁷² ; Ruiz et al. ¹⁸	N/A
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Software and algorithms

serialEM	Mastronarde et al. ⁷³	https://bio3d.colorado.edu/SerialEM/
MotionCor1.3 (MotionCor2 version 1.3.0)	Zheng et al. ⁷⁴	https://msg.ucsf.edu/software
CtfFind4	Rohou et al. ⁷⁵	https://grigoriefflab.umassmed.edu/ctffind4
crYOLO	Wagner et al. ⁷⁶	https://cryolo.readthedocs.io/en/stable/
RELION 5.0	Zivanov et al. ⁷⁷	https://www2.mrc-lmb.cam.ac.uk/relion/index.php/Main_Page
cryoSPARC	Punjani et al. ⁷⁸	https://cryosparc.com/
Blush	Kimanius et al. ⁷⁹	https://relion.readthedocs.io/en/latest/Whats-new.html
EMready	He et al. ⁸⁰	https://sbgrid.org/software/titles/emready
AlphaFold	Jumper et al. ⁸¹	https://alphafold.ebi.ac.uk/
UCSF Chimera	Pettersen et al. ⁸²	https://www.cgl.ucsf.edu/chimera/
UCSF ChimeraX	Meng et al. ⁸³	https://www.cgl.ucsf.edu/chimerax/
COOT	Emsley et al. ⁸⁴	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
PHENIX	Afonine et al. ⁸⁵	https://phenix-online.org/
MolProbity	Chen et al. ⁸⁶	http://molprobity.biochem.duke.edu/
PyMOL	Schrödinger	https://pymol.org/
Clustal Omega 1.2.4	Sievers et al. ⁸⁷	http://www.clustal.org/omega/
FigTree 1.4.4	Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, United Kingdom	http://tree.bio.ed.ac.uk/software/figtree/
CHARMM-GUI	Jo et al. ⁸⁸	https://www.charmm-gui.org/ Accessed on March 31st, 2023.
NAMD 3.0alpha12	Phillips et al. ⁸⁹	https://www.ks.uiuc.edu/Research/namd/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DIA-NN 1.8.1	Demichev et al. ⁹⁰	https://github.com/vdemichev/DiaNN/releases/tag/1.9.2
BioRender	N/A	https://www.biorender.com/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and growth conditions

The strains used in this study are listed in Table S2. *yedD* and *lptD* deletion mutants were constructed by transferring the $\Delta yedD::kan$ allele from the corresponding Keio collection mutant^{36,64} or the $\Delta lptD2::kan$ allele from the MC4100 *ara+* $\Delta lptD2::kan$ pET23/42-*lptD* strain⁶⁶ to appropriate backgrounds^{37,39,41–43,61,62} by P1 transduction.⁹¹ When needed, pCP20 was used to excise the *kan* cassette.^{36,64,69} Transfer of the alleles and excision of the resistance cassette were verified by PCR using primer pairs binding to regions upstream and downstream of the corresponding genes (Listed in Table S4).

To construct strains expressing LptA or LptA_{Strep} (LptA fused to a C-terminal Strep-tag II) from a plasmid in place of endogenous LptA, the following procedure was used. First, the *E. coli* K12 MG1655 strain was transformed with the pSIM19 plasmid (a pSIM5 derivative)⁶⁸ and with a pET23/42 plasmid⁷⁰ carrying either *lptA* or *lptA*_{Strep} (pAG1193 or pAG1221, respectively; see below). The strains were then submitted to λ -red recombineering^{92–94} to replace the chromosomal region from the +35 bp position within *lptA* to the start codon of *lptB* by a *kan* cassette. The PCR product used for recombineering, that contained a *kan* cassette flanked by 40 bp homology regions to the 5' and 3' of the target sequence, was obtained using the AG636 and AG637 primers and the pKD4 plasmid⁶⁴ as a template. Recombinants were selected on kanamycin-containing LB-agar at 30°C and pSIM19 was eliminated by growing selected colonies at 37°C. Replacement of the target sequence by the *kan* cassette was verified by sequencing using primers AG617 and AG618, that bind to regions upstream and downstream of the *lptA* gene. The resulting strains, AG1732 and AG1733, that lack T7 polymerase, produce LptA or LptA_{Strep}, respectively, at low levels from the pET23/42 plasmid, that is driven by a leaky T7 promoter.

To increase the efficiency of *in vivo* photo-crosslinking (unpublished data), we used the AG762 strain, which was constructed as follows. First, the *cat-sacB* cassette, encoding chloramphenicol acetyltransferase (CAT) and SacB, a protein conferring sensitivity to sucrose, was moved from the SEN860 strain^{63,67} to the AG320 strain by P1 transduction. The resulting strain (AG745) was transformed with the pSIM19 plasmid⁶⁸ (a pSIM5 derivative) and submitted to λ -red recombineering^{92–94} to replace *cat-sacB* by a modified *ompA* sequence (*ompA*_{Pal}) encoding OmpA with its 289GIPADK₂₉₄ motif replaced by 137GVSAADQ₁₄₂ from Pal. The PCR product used for this λ -red recombineering step (sharing a 40 base pair homology to the 5' and 3' untranslated regions of *ompA* at its 5' and 3' ends, respectively) was obtained by amplifying *ompA*_{Pal} without its 3' 6×His-tag coding sequence and with a stop codon using primers JLEo42 and JLEo22 and the pPR11_{Pal} plasmid as template. Recombinants were selected on sucrose-containing media (5% sucrose, no NaCl) at 30°C for 16 h. To eliminate pSIM19, the selected colonies were grown at 37°C for 16 h. Loss of the *cat-sacB* cassette in the resulting AG762 strain was verified by positive (sucrose resistance) and negative (chloramphenicol sensitivity) selection and the presence of *ompA*_{Pal} verified by sequencing using primers PL193 and PL194.

Unless otherwise indicated, cells were grown in lysogeny broth (LB) at 37°C, and when necessary, the growth media were supplemented with ampicillin (100–200 µg/mL, from Sigma-Aldrich), chloramphenicol (25 µg/mL, from Sigma-Aldrich), kanamycin (50 µg/mL, from Sigma-Aldrich), or spectinomycin (100 µg/mL, from Sigma-Aldrich).

METHOD DETAILS

Plasmid construction

The plasmids and primers used in this study are listed in Tables S3 and S4, respectively.

To express and purify YedD_{6×His} from the cytoplasm for antibody production, base pairs 49 to 411 of the *yedD* coding sequence were amplified from the pAG310 plasmid using primers AG188 and AG189. The PCR product was then cloned into the pET21a plasmid (Novagen) using NdeI and XhoI restriction sites, yielding plasmid pAG376. This plasmid allowed IPTG-inducible expression of YedD without its signal sequence and without the first cysteine of its mature sequence, with a 6×His-tag fused at its C terminus.

To construct the pLptA and pLptA_{Strep} plasmids (pAG1193 and pAG1221, respectively), the *lptA* coding sequence was amplified from chromosomal DNA (*E. coli* MG1655) using primer pairs AG582 and AG583, which resulted in amplification of *lptA* without a fused tag sequence, or AG582 and AG623, which resulted in the fusion of a Strep-tag II coding sequence at the 3' end of *lptA*. The PCR products were subsequently cloned into the pET23/42 plasmid⁷⁰ by using NdeI and XhoI restriction sites.

The pLT1 plasmid, allowing IPTG-inducible expression of LptE_{Strep} and YedD_{6×His}, was constructed as follows. First, the *lptE* coding sequence was amplified from *E. coli* MG1655 chromosomal DNA using primers AG571 and AG573, resulting in the fusion of a Strep-tag II coding sequence at the 3' end. The PCR product was then cloned in the Multiple Cloning Site 1 (MCS1) of the pCDFDuet-1 plasmid (Novagen) by using the NcoI and BamHI restriction sites, yielding plasmid pAG1129. Subsequently, the *yedD* allele was

amplified from *E. coli* MG1655 chromosomal DNA with primers LT27 and LT28, resulting in the fusion of a 6×His coding sequence at the 3' end. The PCR product was then cloned by NEBuilder HiFi DNA assembly method (NEB) into the MCS2 of the pAG1129 plasmid digested by NdeI and XhoI restriction enzymes.

Amber mutant variants of YedD (listed in Table S3) were constructed by site-directed mutagenesis using the pAG310 plasmid as a template and the primer pairs listed in Table S4.

The pPR11_{Pal} plasmid, that was used for the creation of the AG762 strain, was constructed as follows. To replace the ₂₈₉GIPADK₂₉₄ motif of OmpA by ₁₃₇GVSADQ₁₄₂ from Pal, site-directed mutagenesis was performed using primers pal_GVSADQ_F and pal_GVSADQ_R and the pPR11 plasmid as template to yield plasmid pPR11_{Pal}.

The pAG776 plasmid, a modified version of pTrc99A,⁷² was constructed as follows. To introduce an NdeI restriction site in the MCS region of the pTrc99A plasmid, primers AG363 and AG364 were used to perform site-directed mutagenesis. The resulting plasmid (pAG720) was used as a template to remove the endogenous NdeI restriction site of the pTrc99A plasmid (located outside of the MCS) by using primers AG382 and AG383 to perform another round of site-directed mutagenesis, yielding plasmid pAG776.

To construct the IPTG-inducible expression plasmids pYedD_{Strep} and pYedD_{W70G Q71G R72G-Strep}, the *yedD* coding sequence was amplified from chromosomal DNA (MG1655) using primers NCAo26 and AG235, which resulted in the fusion of a Strep-tag II coding sequence at the 3' end. The PCR product was subsequently cloned into pAG776 using NdeI and XbaI restriction sites, generating plasmid NCAp42 (pYedD_{Strep}). Subsequently, primers AG693 and AG694 and plasmid NCAp42 (as a template) were used to perform site-directed mutagenesis on the *yedD* coding sequence to change amino acid residues 70 to 72 to glycines, generating plasmid pAG1559 (pYedD_{W70G Q71G R72G-Strep}).

The pAM238 complementation plasmids constitutively expressing YedD or YedD_{W70G Q71G R72G} (without tag) were constructed as follows. We first amplified the *yedD* coding sequence in addition to a 49 bp upstream region from the start codon (to include a ribosomal binding site) from the chromosome of strain MG1655 using primers AG175 and AG176. The PCR product was then cloned into pAM238⁷¹ by using KpnI and PstI restriction sites, generating plasmid pAG310 (pYedD). Subsequently, site directed mutagenesis was performed using primers AG693 and AG694 and plasmid pAG310 (as a template) to introduce mutations in the *yedD* coding sequence to change amino acids 70 to 72 into glycines, yielding the pAG1342 plasmid (pYedD_{W70G Q71G R72G}).

LptD variants used in Figure 4 (listed in Table S3) were constructed by site-directed mutagenesis, using plasmids pET23/42-*lptD* and pET23/42-*lptD*_{ΔD57ΔK60} (pLptD_{bridge}) as templates and the primer pairs listed in Table S4.

Pull-down of LptA_{Strep} interacting partners

To perform affinity purification of LptA_{Strep}, overnight cultures of AG1732 (control strain expressing LptA without a tag from the pET23/42 plasmid) and of AG1733 (expressing LptA_{Strep} from the pET23/42 plasmid) were diluted 100-fold into 2 L of fresh LB supplemented with 200 μg/mL ampicillin (Sigma-Aldrich) and cultures were grown at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.6. Cells were then pelleted, resuspended in 25 mL of lysis buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA pH 8, 50 μg/mL DNase I, and 100 μg/mL lysozyme) supplemented with one protease inhibitor tablet (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche) and disrupted by two passes through a French press at 1,500 psi. The resulting cell lysates were centrifuged at 5000 g and 4°C for 10 min to remove unbroken cells and the supernatant was centrifuged at 180,000 g and 4°C for 1 h to pellet membranes. The membrane pellets were solubilized at 4°C for 1 h with 25 mL of solubilization buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA pH 8, 10% glycerol, 5 mM MgCl₂, and 1% *n*-dodecyl-β-D-maltopyranoside (DDM, Anatrace)). Solubilized membranes were centrifuged again at 180,000 g and 4°C for 1 h and the supernatants were incubated for 30 min at 4°C with 2 mL of Strep-Tactin Sepharose resin (IBA Lifesciences) pre-equilibrated with 4 mL of extraction buffer. The samples were then loaded onto 10-mL disposable columns (Pierce) and the resin washed with 60 mL of wash buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA pH 8, 10% glycerol, and 0.05% DDM). Elutions were performed with 6 mL of elution buffer (wash buffer supplemented with 10 mM D-Desthiobiotin from Millipore) and eluates were concentrated to a final volume of 50 μL using Vivaspin 500 centrifugal concentrators with a 5-kDa cutoff (Sartorius). 12.5 μL of 5× concentrated SDS sample buffer (SDS sample buffer: 2% SDS, 10% glycerol, 60 mM Tris-HCl pH 7.4, 0.01% bromophenol blue) were added to each eluate, samples were boiled for 10 min and used for SDS-PAGE analysis. Protein bands were then either stained with PageBlue Protein Staining Solution (Thermo Fisher Scientific) and excised for MS analysis or submitted to immunoblot analysis.

Pull-downs of YedD_{Strep} and YedD_{W70G Q71G R72G-Strep} interacting partners

To perform affinity purification of YedD_{Strep} and YedD_{W70G Q71G R72G-Strep}, cultures of NCAs137 and AG1572 were grown at 37°C after diluting overnight cultures 100-fold into 500 mL of fresh LB supplemented with 200 μg/mL ampicillin (Sigma-Aldrich). Protein expression was induced with 500 μM IPTG (Promega) at an OD₆₀₀ of 0.6 and cultures were further grown for 3 h at 37°C. Cells were then pelleted, resuspended in 25 mL of PBS supplemented with one protease inhibitor tablet (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche), and disrupted by two passes through a French press at 1,500 psi. The resulting cell lysates were centrifuged at 40,000 g and 4°C for 40 min to pellet membranes. Membrane pellets were first solubilized for 1 h at 4°C in 20 mL of inner membrane (IM) solubilization buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, and 0.5% *N*-Lauroylsarcosine (Sigma-Aldrich)), followed by centrifugation at 40,000 g and 4°C for 40 min. The resulting membrane pellets were further solubilized for 16 h at 4°C in 20 mL of outer membrane (OM) solubilization buffer (20 mM Tris-HCl pH 7.5, 300 mM NaCl, and 1% DDM (Inalco Pharmaceuticals)), followed by one last centrifugation step at 40,000 g and 4°C for 40 min to remove insoluble material. The supernatants (inputs) were incubated

for 30 min at 4°C with 0.5 mL of Strep-Tactin Sepharose resin (IBA Lifesciences) pre-equilibrated with 1 mL of OM solubilization buffer. The samples were then loaded onto 10-mL disposable columns (Pierce) and the resin washed with 15 mL of wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% DDM). The proteins were eluted with 2 mL of elution buffer (wash buffer supplemented with 2.5 mM D-Desthiobiotin from Millipore) and eluates were concentrated to a final volume of ~300 μ L using Vivaspin 500 centrifugal concentrators with a 5-kDa cutoff (Sartorius). An appropriate amount of 5 $\times$ concentrated SDS sample buffer was added to input samples and to concentrated eluates, samples were boiled for 10 min and used for SDS-PAGE analysis, followed by immunoblotting.

Purification of the LptDE-YedD complex

The pLT1 plasmid was co-transformed with pET23/42-*lptD* in C43 (DE3) cells.⁶⁵ An overnight culture of the cells was diluted 100-fold into LB containing 0.2% glucose, 200 μ g/mL ampicillin (Sigma-Aldrich) and 100 μ g/mL spectinomycin (Sigma-Aldrich) and cultures were grown at 26°C until the OD₆₀₀ reached 0.8. Protein expression was then induced by addition of 0.1 mM IPTG (Promega) and the cultures were further grown for 20 h at 26°C. Cells were harvested and pellets were frozen. Pellets were resuspended in purification buffer (20 mM Tris-HCl pH 7, 150 mM NaCl) containing 1mM EDTA and cComplete EDTA-free protease inhibitor tablets (Roche), and the cells were disrupted by three passes through a French press at 1,500 psi. Lysates were directly centrifuged at 100,000 *g* for 1 h at 4°C and supernatants were discarded. Pellets were resuspended in purification buffer containing 1% *n*-dodecyl- β -D-maltopyranoside (DDM, Anatrace) and the membranes solubilized at 4°C overnight on a roller. The following day, solubilized samples were clarified by centrifugation at 40,000 *g* for 40 min at 4°C. Supernatants were kept at 4°C while pellets were resuspended a second time in purification buffer containing 1% DDM and incubated at 4°C with a magnetic stirrer for 2 h. After clarification, supernatants from this second membrane solubilization step were pooled with the ones from the first step and diluted with purification buffer to reach a concentration of 0.2% DDM. The resulting sample was then loaded on a 5-mL Strep-Tactin superflow high capacity cartridge (IBA Lifesciences) using an ÄKTA protein purification system (Cytiva) and washed with at least 10 column volumes (CV) of purification buffer containing 0.2% DDM. The complex was then eluted with 5 CV of 2.5 mM D-Desthiobiotin (Millipore) in purification buffer containing 0.2% DDM. A second purification step was then performed, to enrich the complexes containing the YedD protein. The elution fractions of the Strep-Tactin purification were pooled and incubated with 500 μ L of Ni-NTA resin (Thermo Fisher Scientific) for 1 h at 4°C on a roller. The beads were washed with at least 10 CV of purification buffer containing 0.2% DDM, and eluted with 5 CV of elution buffer 1 (20 mM Tris-HCl pH 7, 150 mM NaCl, 0.03% DDM, 200 mM imidazole (Millipore)) followed by 2.5 CV of elution buffer 2 (20 mM Tris-HCl pH 7, 150 mM NaCl, 0.03% DDM, 400 mM imidazole). Fractions containing stoichiometric ratios of the LptD, LptE and YedD proteins were pooled and concentrated using a Vivaspin 4 with a 30-kDa cutoff (Sartorius) and sent for structural analysis by cryo-electron microscopy.

Purification of YedD_{6 $\times$ His}

An overnight culture of AG377 was diluted 100-fold into 6 $\times$ 1 L of fresh LB supplemented with 200 μ g/mL ampicillin (Sigma-Aldrich), and cultures were grown at 37°C. Protein expression was induced with 1 mM IPTG (Promega) at an OD₆₀₀ of 0.6 and cultures were further grown for 3 h at 37°C. Cells were pelleted, resuspended in 30 mL of Buffer A (50 mM NaPi pH 8, 300 mM NaCl) and disrupted by two passes through a French press at 1,500 psi. The resulting cell lysate was clarified by centrifugation at 30,000 *g* and 4°C for 45 min and the supernatant loaded on three serially-connected 1-mL HisTrap HP columns (Cytiva) previously equilibrated with Buffer A using an ÄKTA protein purification system (Cytiva). The columns were washed with 40 mL of Buffer A, and YedD_{6 $\times$ His} was eluted by applying a 60-mL linear gradient of imidazole (from 0 to 300 mM, Millipore) in Buffer A. The fractions containing YedD_{6 $\times$ His} were pooled, concentrated using a 5-kDa cutoff Vivaspin 15 device (Sartorius) and desalted using a PD-10 column (Cytiva) equilibrated with 50 mM NaPi pH 8, 150 mM NaCl. Size-exclusion chromatography using the ÄKTA purification system was then performed by loading Ni-NTA-purified YedD_{6 $\times$ His} on a 120-mL HiLoad 16/60 Superdex 75 PG column (Cytiva) previously equilibrated with 20 mM HEPES-KOH pH 7.4, 100 mM NaCl. The resulting YedD_{6 $\times$ His} - containing fractions were pooled and concentrated using a 5-kDa cutoff Vivaspin 15 device (Sartorius), and the size-exclusion chromatography step was repeated a further two times.

Electron microscopy data collection

3 μ L of LptDE-YedD sample (~0.5 mg/mL) was spotted on a glow-discharged R1.2/1.3 copper 400 mesh grid (Quantifoil). Grids were blotted for 3–4 s (2-side), 90% humidity and flash-frozen in liquid ethane using a CP3 plunger (Gatan). Frozen grids were then loaded onto a 300-kV CRYO ARM 300 (JEM-Z300FSC) Field Emission Cryo-Electron Microscope (JEOL) equipped with a K3 Summit direct electron detector (Gatan). Image stacks were recorded in counting mode with a cumulative electron dose of 60 electrons per $\text{\AA}^2$ spread over 60 frames, at 60K magnification, using serialEM.⁷³ Energy filter was inserted and the defocus ranges from 0.8 to 2.0 μ m. Data collection was accelerated using multi-hole shot scheme where 3 shots per hole and nine holes per stage move was applied.

Data processing and 3D reconstruction

Frame motion correction was done using MotionCorr1.3⁷⁴ and defocus values were estimated using CtfFind4.⁷⁵ Dose-weighting scheme was applied. Particle picking was done in crYOLO using a pre-trained model from a small subset of 20 micrographs with manually picked particles.⁷⁶ Particle extraction was done in RELION (3 $\times$ binned),⁷⁷ and particle stack file was then exported to

cryoSPARC⁷⁸ for multiple rounds of 2D classification. A subset of clean particles was used for 3D ab-initio reconstruction in cryo-SPARC, following by few rounds of heterogeneous refinement. LptDE-YedD representing 3D class was then re-imported into RELION for a new particle extraction without binning. One round of 3D classification with Blush⁷⁹ was done in RELION. The final LptDE-YedD particle subset was then imported into cryoSPARC for non-uniform refinement, with option of minimize over per-particle scale at each iteration of refinement and CTF refinement. The reconstruction map was further sharpened, and density modified using EMready.⁸⁰

Model building, refinement and validation

First LptDE-YedD model was done by docking AlphaFold⁸¹ predicted structures of *E. coli* K12 LptD/LptE/YedD into the reconstruction map using UCSF Chimera.⁸² Manual model building was carried out in COOT.⁸⁴ Refinement was performed using PHENIX real-space refinement⁸⁵ in combination with manual rebuilding using COOT. The final map quality and fit to the map were inspected using MolProbity.⁸⁶ Three-dimensional reconstruction was displayed, and figures were prepared in UCSF Chimera,⁸² UCSF ChimeraX⁸³ and PyMOL (<https://pymol.org/2/>). Data collection and refinement statistics are found in Table S1.

Phylogenetic analysis

A BLASTP search in the nr database carried out on November 22nd, 2022 using the sequence of *Escherichia coli* YedD (UniProt code P31063) as input, followed by manual curation, produced 2466 complete sequences of YedD from different species. These sequences were aligned using Clustal Omega⁸⁷ version 1.2.4 and the phylogenetic tree produced with the default neighbor-joining algorithm. The figure was generated with FigTree version 1.4.4.

Interactive molecular-dynamics flexible fitting of the cryo-EM model

For the structural analysis of the LptE-YedD interaction, the missing residues (169–193) of LptE from the cryo-EM structure were completed with those from the AlphaFold model (https://alphafold.ebi.ac.uk/files/AF-P0ADC1-F1-model_v4.pdb).

Molecular dynamics simulation of the LptDE-YedD complex

The cryoEM structure of the complex LptD-LptE-YedD with the coordinates of residues 169–193 of LptE completed using the AlphaFold2 prediction (https://alphafold.ebi.ac.uk/files/AF-P0ADC1-F1-model_v4.pdb). Cliquez ou appuyez si vous faites confiance à ce lien.">https://alphafold.ebi.ac.uk/files/AF-P0ADC1-F1-model_v4.pdb) was prepared using the CHARMM-GUI webserver⁸⁸ and the standard protocol for Membrane Builder/Bilayer Builder and CHARMM force field. The prepared system containing 199,051 atoms (including 56 LPS molecules for the outer leaflet and 120 PPPE, 32 PVPG and 8 PVCL2 lipids for the inner leaflet of the membrane) was equilibrated using NAMD⁸⁹ with the standard CHARMM-GUI protocol for membrane proteins, then a production run was carried out for 100 ns. The final frame of the simulation is presented in Figure S5, showing a direct interaction between LptE and YedD.

Mass spectrometry analysis

Protein bands were visualized using the PageBlue Protein Staining Solution (Thermo Fisher Scientific) and in-gel digested with Trypsin (Promega). Peptides were extracted with 0.1% trifluoroacetic acid (TFA) in 65% acetonitrile (ACN) and dried in a speedvac. Peptides dissolved in solvent A (0.1% TFA in 3.5% ACN) were directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Fisher Scientific, Waltham, United States) and eluted in backflush mode. Peptide separation was achieved using a reversed-phase analytical EasySpray column (Acclaim PepMap RSLC C18, 0.075 mm × 250 mm, Thermo Fisher Scientific, Waltham, United States) with a 90-min linear gradient of 4%–27% solvent B (0.1% TFA in 80% ACN) for 40 min, 27%–50% solvent B for 10 min, 50%–95% for 3 min and holding at 95% for the last 10 min at a constant flow rate of 300 nL/min on an Ultimate 3000 RSLC nanoHPLC system (Thermo Fisher Scientific, Waltham, United States). The peptides were analyzed by an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific, Waltham, United States) with enabled advanced peak determination (APD). The peptides were subjected to an EasySpray ionization source, followed by MS/MS in the Exploris 240 coupled online to the HPLC. Intact peptides were detected in the Orbitrap at a full width at half-maximum resolution of 30,000 (Full width at half maximum; FWHM) and MS/MS spectra were acquired in the Orbitrap after stepped higher energy collisional dissociation (HCD) fragmentation at 22%, 26% and 30% of maximum. A data-independent (DIA) procedure of MS/MS scans was applied for the precursor ions within an m/z range of 500–740 and an m/z isolation window of 4 Da at a resolution of 60,000 (FWHM). MS1 spectra were obtained with an automatic gain control (AGC) target of 1.2×10^6 ions and a maximum injection time of 55ms. MS2 spectra were acquired with an AGC target of 1.5×10^5 ions and a maximum injection time set to auto. For MS2 scans, the m/z scan range was set at 145 to 1450. DIA MS dataThermo.raw spectra files for all samples were imported into DIA-NN⁹⁰ using the Thermo MS File Reader (Thermo Fisher Scientific, Waltham, United States). In addition to default options, the following options were used: FASTA digest for library-free search/library generation; Deep learning-based spectra, RTs and IMs prediction; Maximum number of variable modifications = 1, Modifications = N-term M excision, C carbamidomethylation, Ox(M); Unrelated runs; Neural network classifier = Double-pass mode; Quantification strategy = Any LC (high accuracy); Precursor FDC (%) = 1.0.

In vivo photo-crosslinking experiments

To identify interactions between YedD and LptDE, the AG762 strain was co-transformed with pEVOL and pAG310 with an amber codon (TAG) introduced at a specific position in the *yedD* sequence. Overnight cultures of the strains were diluted 1000-fold in 3.5 mL fresh LB supplemented with 25 μ g/mL chloramphenicol (Sigma-Aldrich), 100 μ g/mL spectinomycin (Sigma-Aldrich), 0.8 mM *p*-benzoyl-L-phenylalanine (pBpa; Bachem) and 0.2% L-(+)-Arabinose (Sigma-Aldrich) and the cultures were grown at 37°C until the OD₆₀₀ reached ~0.75. 1 mL of each culture was split in two, the first half being set aside to serve as a non-UV treated sample (UV -), while the second half was exposed to UV light (λ = 365 nm) for 10 min at room temperature (UV + sample). 10% w/v trichloroacetic acid was added to UV - and UV + samples, followed by incubation on ice for 30 min, and centrifugation at 16,000 *g* and 4°C for 10 min. The resulting pellets were rinsed with 1 mL ice-cold acetone, then pelleted again at 16,000 *g* and 4°C for 10 min. Finally, acetone was discarded, the pellets were dried, resuspended in 50 μ L of SDS sample buffer, boiled for 10 min, and used for immunoblot analysis.

Efficiency of plating assays

To evaluate the fitness and vancomycin sensitivity of *lptD_{wt}*, *lptD_{wt} Δ yedD*, *lptD_{bridge}* and *lptD_{bridge} Δ yedD* cells, overnight cultures of strains HC340, HC509, AG992 and AG993 were diluted 1000-fold into 5 mL of fresh LB or M9 minimal medium (MP Biomedicals) supplemented with 0.2% glucose, 1 mM MgSO₄ and 100 μ M of CaCl₂. Both media were supplemented with 200 μ g/mL ampicillin (Sigma-Aldrich) and cultures were grown at 37°C until the OD₆₀₀ reached ~0.5. The cultures were then 10-fold serially diluted and spotted respectively on LB-agar or supplemented M9 minimal medium-agar plates, both containing 200 μ g/mL ampicillin. The serially-diluted, LB-grown cultures were also spotted on 200 μ g/mL ampicillin-containing LB-agar plates additionally supplemented with 200 μ g/mL vancomycin (Alfa Aesar). All plates were then incubated overnight at 37°C.

To evaluate the ability of plasmid-encoded YedD or YedD_{W70G Q71G R72G} to restore the fitness of the *lptD_{bridge} Δ yedD* strain, overnight cultures of strains of AG1034, AG1035 and AG1445 were diluted 1000-fold into 5 mL of fresh LB supplemented with 100 μ g/mL spectinomycin (Sigma-Aldrich) and 200 μ g/mL ampicillin (Sigma-Aldrich). Cultures were grown at 37°C until the OD₆₀₀ reached ~0.5, were 10-fold serially diluted and spotted on LB-agar plates supplemented with 100 μ g/mL spectinomycin and 200 μ g/mL ampicillin. The plates were then incubated overnight at 37°C.

The fitness and vancomycin sensitivity of *lptB_{wt}*, *lptB_{wt} Δ yedD*, *lptB_{E86Q}*, *lptB_{E86Q} Δ yedD*, *lptB_{R150A}*, *lptB_{R150A} Δ yedD*, *lptB_{R198A}* and *lptB_{R198A} Δ yedD* cells were evaluated by diluting overnight cultures of strains AG1820, AG1821, AG1822, AG1823, AG1824, NR2101, NR2840, NR2952, NR4446 and NR4978 1000-fold into 5 mL of fresh LB supplemented with 200 μ g/mL ampicillin. The cultures were grown at 37°C until the OD₆₀₀ reached ~0.5, then 10-fold serially diluted and spotted on LB-agar plates containing 200 μ g/mL ampicillin. The serially-diluted cultures were also spotted on 200 μ g/mL ampicillin-containing LB-agar plates additionally supplemented with 50 μ g/mL or 75 μ g/mL vancomycin. All plates were then incubated overnight at 37°C.

To evaluate LPS modification in *lptD_{wt}*, *lptD_{wt} Δ yedD*, *lptD_{bridge}* and *lptD_{bridge} Δ yedD* cells, as well as LptD levels and oxidation status in *lptD_{wt}*, *lptD_{wt} Δ yedD*, *lptD_{bridge}*, *lptD_{bridge} Δ yedD* and *lptD₄₂₁₃* cells, overnight cultures of strains HC340, HC509, AG992, AG993 and NR698 were diluted 1000-fold into 5 mL of fresh LB. The medium was supplemented with 200 μ g/mL ampicillin (except for the NR698 strain that carries the *lptD₄₂₁₃* allele on the chromosome and not on a plasmid) and cultures were grown at 37°C until the OD₆₀₀ reached ~0.5. 10% w/v trichloroacetic acid was then added to 1 mL of each culture, and the samples processed as described in the *in vivo* photo-crosslinking experiments section. The pellets were resuspended in 50 μ L of SDS sample buffer, boiled for 10 min and the resulting samples used for immunoblot analysis. To visualize reduced LptD levels, 5% β -Mercaptoethanol was added to the SDS sample buffer prior to boiling.

SDS-PAGE and immunoblot analysis

Proteins were separated on NuPAGE 10% Bis-Tris gels using MOPS buffer (visualization of LptD folding intermediates), NuPAGE 12% Bis-Tris gels using MES buffer (visualization of LPS modification) or NuPAGE 4%–12% Bis-Tris gels using MES buffer (all other experiments). Gels and buffers were purchased from Thermo Fisher Scientific. Proteins were then detected with the PageBlue Protein Staining Solution (Thermo Fisher Scientific), by Coomassie staining, or by immunoblot analysis. For immunoblot analysis, proteins were transferred to nitrocellulose membranes using the iBlot or iBlot 2 transfer devices (Invitrogen). The membranes were blocked for 30 min to 1 h at room temperature with shaking in 5% skim milk in TBS-T (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.1% Tween 20). Incubation with the primary antibody diluted in a solution of 1% skim milk in TBS-T was performed for 1 h at room temperature with shaking. The anti-LptC antibody was diluted 1:1,000, the anti-YedD, anti-LptE and anti-Strep-tag II antibodies 1:5,000, the anti-LPS core antibody 1:1,000 or 1:5,000 depending on the antibody batch used, and the anti-LptD antibody 1:20,000. After washing with TBS-T, the membranes were incubated for 1 h at room temperature with shaking in a solution of 1% skim milk in TBS-T containing an HRP-linked anti-rabbit IgG diluted 1:10,000 or an HRP-linked anti-mouse IgG diluted 1:5,000. After washing with TBS-T, labeled proteins were detected by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific or Immobilon Western Chemiluminescent HRP Substrate, Millipore) and visualized using X-ray films (Fujifilm) or the Amersham ImageQuant 800 imaging system.

Antibodies

Polyclonal antibodies directed toward YedD were generated by immunizing rabbits with YedD_{6×His} (CER Group, Marloie, Belgium). The serum was further purified by affinity to YedD_{6×His} in denaturing conditions as follows. 1.5 mg of YedD_{6×His} was conjugated in a denaturing buffer (0.5 M NaHCO₃, 0.5 M NaCl, 4 M GdnHCl, pH 8.2) to 2 mL NHS-Activated Sepharose 4 Fast Flow resin (Cytiva). The resin was resuspended in 0.1 M Tris, 0.5 M NaCl, pH 8.5 and packed in an HR5/5 column (Pharmacia). The resin was washed with a first buffer (0.1 M Tris, 0.5 M NaCl, pH 8.5) followed by a second (0.1 M NaAc, 0.5 M NaCl, pH 4). 40 mL of serum containing polyclonal antibodies against YedD_{6×His} were applied onto the affinity column. After washing with PBS, bound antibodies were eluted in 50 mM glycine-HCl, 150 mM NaCl, pH 2.7. The eluate was immediately neutralized with 1 M Tris pH 8. After concentrating the eluate on a 10-kDa cutoff column and desalting on a Hi-trap column (Cytiva), about 2.52 mg of the purified YedD antibodies were obtained. The anti-LptC, LptD, and LptE antibodies were generous gifts from A. Polissi (University of Milano), S. Matsuyama (Rikkyo University), and D. Kahne (Harvard University), respectively. To detect lipopolysaccharide (LPS), a monoclonal antibody directed toward the lipopolysaccharide core was used (mAb WN1 222-5, Hycult Biotech). To detect YedD_{Strep} and YedD_{W70G Q71G R72G-Strep}, a monoclonal antibody directed toward Strep-tag II (StrepMAB-Classic, IBA Lifesciences) was used. As secondary antibodies, an HRP-linked anti-rabbit IgG (NA934, Cytiva) or an HRP-linked anti-mouse IgG (A4416, Sigma-Aldrich) were used.

Viability assays

Plasmids encoding LptD or LptD variants (listed in [Table S3](#)) were transformed into NR754 and AG1391 strains. The ability of the plasmid-encoded LptD mutants to sustain viability was then assessed by performing P1 transduction on the resulting strains to determine whether a null $\Delta lptD2::kan$ allele could replace the chromosomal *lptD* gene. The number and size of colonies obtained following P1 transduction was recorded for each strain and the replacement of chromosomal *lptD* by the null $\Delta lptD2::kan$ allele, when possible, was verified by PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

There are no quantification or statistical analyses to include in this study.