Structural basis for outer membrane lipopolysaccharide insertion

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Lipopolysaccharide (LPS) is essential for most Gram-negative bacteria and plays crucial roles in protection of the bacteria from harsh environments and toxic compounds, including antibiotics[1,2]. Seven LPS transport proteins (i.e. Lpt A – G) form a trans-envelope protein complex responsible for the transport of LPS from the inner membrane to the outer membrane, the mechanism for which is poorly understood[3,4]. Here we report the first crystal structure of the unique integral membrane LPS translocon LptD/E complex. LptD forms a novel 26-stranded β-barrel, which is the largest β-barrel reported to date. LptE adopts a roll-like structure located inside the barrel of LptD to form an unprecedented two-protein "barrel and plug" architecture[5]. The structure, molecular dynamics simulations and functional assays suggest that the hydrophilic O-antigen and the core oligosaccharide of the LPS may pass through the barrel and the lipid A of the LPS may be inserted into the outer leaflet of the outer membrane through a lateral opening between strands β1 and β26 of LptD. These findings not only help us to understand important aspects of bacterial outer membrane biogenesis but also have significant potential for the development of novel drugs against multi-drug resistant pathogenic bacteria.

All Gram-negative bacteria have an asymmetric outer membrane, in which the inner leaflet consists of phospholipid and the outer leaflet is comprised of lipopolysaccharide (LPS)[1,2]. LPS normally has three components, lipid A, core oligosaccharide and O-antigen (Fig. 1a), forming a large amphipathic macromolecule, which is essential for the viability of most Gram-negative bacteria. Seven LPS transport proteins, abbreviated LptA-LptG, form the trans-envelope protein complex that is responsible for the transport of LPS from the inner membrane to the outer membrane (OM)[3,4,5,6,7]. The ABC transporter complex LptBFGC extracts LPS from the inner membrane and passes it to a periplasmic chaperon LptA. LptA delivers the LPS to the LptD/E complex, which subsequently translocates the LPS from the periplasm into the outer membrane[8,9].

LptD is unable to fold properly in the absence of LptE and the two proteins form a unique "barrel and plug" architecture for LPS transport[5,8,9], the mechanism of which is poorly understood. Additionally, the LPS transport proteins are essential for most pathogenic Gram-negative bacteria, and therefore the LptD/E complex is a particularly attractive drug target[10]. The LptD/E complex is highly conserved in Gram-negative bacteria (Extended data Fig. 1, 2), so a detailed structural model should help us to better understand outer membrane LPS biogenesis. We report here the first X-ray crystallographic structure of the LptD/E complex, which provides a structural basis for LPS translocation across the outer membrane and insertion into the outer leaflet.

In order to obtain the LptD/E protein crystals of *Salmonella typhimurium* strain LT2, the two proteins were co-expressed and subjected to limited proteolysis with α-chymotrypsin (Extended data Fig. 3). The resultant LptD/E complex crystallized in space group I2. The structure of LptD/E was determined by multi-wavelength

anomalous dispersion to 2.86-Ångström resolution, and the final model was refined to R_{work} of 0.28 and R_{free} of 0.30 (Extended data table 1, Supplementary Fig. S1, S2). The LptD model contained residues 226 to 786 and the LptE model consisted of residues 19 to 169 (both numbering includes the signal peptide). Electron density for residues 293-307, 725-728 and 759-770 of LptD could not be observed, suggesting a high degree of flexibility. LptD forms a 26 anti-parallel stranded β-barrel with dimensions of approximately 70 Å in length, 50 Å in width and 50 Å in height (Fig. 1b - f), while LptE forms a roll-like structure (Fig. 2a) with a long axis of 63 Å and a diameter of 21 Å at the widest point. Around three-quarters of LptE are located inside the β-barrel of LptD, with the remainder in the periplasm. The barrel is closed by strands β1 and β26 with the N terminus in the periplasm and the C terminus hidden inside the periplasmic side of the barrel (Fig. 1b, f). The N-terminus of LptE is acyl-modified and is in the perfect position for insertion into the inner leaflet of outer membrane (Fig. 1c). The largest previously reported single-protein barrels, PapC and FimD, comprise 24 anti-parallel β-strands. These barrels are completely blocked by a plug formed by the middle domain of PapC or FimD[11,12]. In contrast, LptD forms a 26-stranded β-barrel and is "plugged" by another protein, LptE. To our knowledge, LptD is the largest single-protein outer membrane β-barrel to date, and also the only barrel structure that has been demonstrated to use another protein as a "plug"[5,13].

To assess any structural changes in LptE due to its association with LptD, we superimposed the LptE structure with the four available LptE crystal and NMR structures in the protein data bank (2R76, 3BF2, 4KWY and 2JXP). Although the amino acid identities are as low as 13%, the LptE structures are strikingly superimposable in terms of the α-helices and β-sheets (Fig. 2b, and Extended data Fig. 5). Given the essential roles of LptE in LptD folding and assembly, we predict that

most of the LptD/E complexes of Gram-negative bacteria possess a similar structure to that of the complex from *S. typhimurium* that we present here.

The β-strands in the LptD barrel are linked by loops, which are much longer on the extracellular side than those at the periplasmic side (Fig. 1b). Thirteen loops exist between strands at the extracellular side, namely Lp1 to 13. Most of the loops at the extracellular side are at the surface of the pore. However, loop 4 and 8 are located in the interior of the pore and consist of residues V334-Y354 and V519-S556, respectively (Fig. 1e and Extended data Fig. 6). The LptD/E structure confirms that these two loops are involved in LptD and LptE interactions[5,9,14]. The surface area of monomeric LptE is 9860.8 $Å^2$, of which 3195.4 $Å^2$ is involved in formation of the interface with LptD. Around 37 residues of LptE interact with 52 residues of LptD to stabilize the β barrel (Fig. 2c). Residues A87-T95 of LptE interact with residues in Lp4, Y680 and C-terminal residues of LptD. LptE also interacts with residues T771-M784 on lumen of the LptD barrel through the C-terminal residues (Fig. 2d, e and Extended data Fig. 6). In addition, the LptD/E interactions are enhanced by hydrophobic interactions between the N-terminal residues W21-L23 of LptE and the LptD at the outer surface of the barrel (Fig. 2f). In order to test the structural findings further, we generated single alanine or glycine amino acid substitution mutations, LptE deletions $LptE_{\Delta W21\text{-}L23}$ and $LptE_{\Delta A87\text{-}T95}$, and C-terminal truncations $LptE_{\Delta T170\text{-}N196}$ and $LptD_{\Delta T771\text{-}M784}$ (Supplementary table S1, S2). Functional assays show that all the LptD and LptE mutations can grow in LB medium as well as their wild-types. However, those with deletions $LptE_{\Delta W21\text{-}L23}$, $LptE_{\Delta A87\text{-}T95}$ and $LptD_{\Delta T771\text{-}M784}$ significantly impair cell growth of *E. coli* in LB medium containing 0.5% SDS and 1 mM EDTA, with the exception of $LptE_{\Delta T170\text{-}N196,}$ where the deleted residues are not involved in the LptD/E interaction (Fig. 2g, Extended data table 2, 3). In all cases the

mutated proteins are expressed at similar levels to the wild-type proteins (Fig. 2h, i). We conclude that mutations of $LptE_{\Delta W21\text{-}L23}$, $LptE_{\Delta A87\text{-}T95}$ and $LptD_{\Delta T771\text{-}M784}$ interfere with LptD/E interactions, either causing poor plugging of LptE in the LptD barrel or reduced LptD assembly, thus increasing outer membrane permeability to SDS. These results are consistent with the structural findings and previous reports[5,9,15,16,17].

The LptD protein forms a kidney-shaped pore with a 70 Å by 50 Å (outer side) and 50 Å by 30 Å (inner side) diameter at its widest points (Fig. 1e, f, Fig. 3a, b). The extracellular loops of LptD seal most of the pore but leave a hole at one side of the pore with diameter approximately 15 Å by 10 Å, which is blocked by LptE to close the LptD channel completely (Fig. 3a, b). The lumen of the barrel is highly hydrophilic, similar to the polysaccharide translocons Wza[18] and AlgE[19]. How is the hydrophobic lipid A transported in the barrel of the LptD/E complex? As LptE binds the lipid A component of LPS specifically[14], it is likely that LptE assists lipid A transport inside the LptD barrel. The hydrophobic residues inside the β-jellyroll structures of LptA and LptC have been identified to bind lipid A of LPS[20]. We speculate that hydrophobic residues at the interface between the long α2 and the four β-strands of LptE may play a role in the lipid A interaction inside the LptD barrel (Supplementary Fig. S3). However, a study in *Neisseria meningitidis* suggested that LptE is not directly involved in LPS transport[21]; therefore further investigation is required to clarify the role of LptE.

When viewed from the extracellular face, the β-strands of the barrel are twisted in an anticlockwise fashion relative to the periplasmic side (Fig. 1e, f). The angle of strands β1- 4 is around 30° relative to the plane of the membrane, while that of strands β20-26 is about 67°, showing that strands β1 and β26 are partially separated at the periplasmic side (Fig. 3c, e). This phenomenon has been seen in other outer membrane barrels

FadL[22], OmpW[23] and PagP[24] in which this feature laterally opens the barrel wall for hydrophobic substrate diffusion. There are only five hydrogen bonds between strands β1 and β26 of LptD (Fig. 3d), suggesting that lateral opening of the barrel between these strands is possible. Disulfide bond formation with LptD is essential for LPS translocation[26,27] and this may play a role in any lateral opening of LptD. Intriguingly, C726 and C727 of LptD are located in the flexible loop between strands β24 and β25 at the periplasmic side (Fig. 3c). This loop is in a perfect position for disulfide bond formation with either C31 or C173 in the N-terminal domain of LptD, which is directly linked to strand β1 of LptD.

To test the hypothesis that a lateral opening of LptD is required for the LPS translocation, we generated single/double amino acid substitutions and deletion mutants of *lptD* (Supplementary table S2), and then performed functional assays. The deletion mutation $LptD_{\Delta Q722\text{-}A729}$, removing C726 and C727, results in the death of the *E.coli* cells, consistent with previous reports[26,27]. The structure of LptD suggests that the double amino acid mutation N232C/N757C may form a disulfide bond in the oxidative periplasm (Extended data Fig. 7), which would lock the β1 and β26 strands of LptD together and prevent any lateral opening. Indeed, the double mutation of N232C/N757C is lethal, whereas the single mutations of N232C and N757C and double mutations N232D/N757R and N232Y/N757H of LptD retain the same vitality as the wild-type (Fig. 3f, Extended data table 3). The protein expression level of the double mutation N232C/N757C is similar to that of single mutation N757C (Fig. 3g), strongly indicating that some degree of lateral opening between β1 and β26 of LptD is required for LPS translocation and insertion.

To further investigate the LPS translocation mechanism, molecular dynamics (MD) simulations of LptD/E complex were performed using two distinct approaches. In the

first, high temperature simulations (340K, 350K, 375K and 400K) were performed, which showed that the change in temperature was unable to separate the β1 and β26 strands (Extended data Fig. 8). Therefore a second approach of applying membrane stretch was applied, by simulating under different lateral pressures, from -10 to -100 bar. For pressures above -65 bar, separation of the β1 and β26 strands was observed and the LptD channel opened simultaneously (Extended data Fig. 8), with membrane lysis usually following within 10 ns. While the applied pressures are non-physiological and are unlikely to resemble the true mechanism of gating in LptD/E, the strand separation does reveal that this is the weakest point within the structure and is therefore suggestive of the LPS insertion pathway. Pressures of -50, -75 and -100 bar were also applied to simulations of other outer membrane proteins: BtuB (PDB accession code 2GUF) and FhaC (2QDZ), as well as the N232C/N757C mutant LptD/E complex. In these simulations no perturbation of the barrel architecture was observed prior to bilayer lysis.

Bacterial polysaccharides are transported across the outer membrane by either a Wza-like protein[18], or via a β-barrel protein such as AlgE[19]. The lumen of the LptD/E barrel is highly hydrophilic, a feature of translocases for hydrophilic polymer translocation[18,19]. The O-antigen of lipopolysaccharide of *S. typhimurium* consists of trisaccharide repeat units with short branches of single sugars (Fig. 1a), suggesting that the diameter of the linear polymer is around 13 Å for the O-antigen. Despite being occupied by LptE, the free cavity of LptD still has a diameter of 25 by 15 Å (Fig. 3b), which could easily accommodate the O-antigen. The O-antigen, which may consist of hundreds of saccharide units, has to pass through the LptD barrel, possibly using a similar mechanism to AlgE[19]. How does the LptD/E translocon insert the lipid A of LPS into the outer leaflet of the membrane? The challenge of transporting large

hydrophobic substrates through an aqueous barrel into the outer membrane is formidable. Nevertheless, several other outer membrane protein barrels adopt a lateral opening mechanism to diffuse the hydrophobic molecules to the outer membrane[22-25]. LPS normally contains six fatty acyl chains, which would be 25 Å in length and 5 Å in width (Fig. 1a). Therefore, it would be big enough for the lipid chains of LPS to pass from the 5 Å side to locate the LPS to the outer leaflet of the outer membrane if strands β1 and β26 could open around 7 - 9 Å. The diameter of the free cavity of the LptD barrel with LptE is 25 by 15 Å, which may help to position the 5 Å side of lipid A to the gate between the strands β1 and β26. Once the non-consecutive disulfide bonds form, the LptD N-terminus links to LptA and forms a trans-envelope complex together with other LPS transport proteins from the inner membrane to the outer membrane. We propose that, by using ATP, the LPS is extracted from the inner membrane and passed to LptC[28], LptA[29] and the LptD/E complex sequentially[30], triggering a lateral opening of LptD and opening of the LptD channel and thus promoting the lateral insertion of LPS into the outer membrane through the gate between the strands β1 and β26 of LptD (Fig. 4). The core polysaccharide of the LPS is highly negatively charged and is bridged to neighbouring molecules through divalent cations. This forms the outer membrane permeability barrier, along with the hydrophobic lipid A tails. We expect that once the LPS reaches the cation-rich outer leaflet of outer membrane both hydrophobic and electrostatic components would drive the LPS to correctly insert into the outer leaflet of the outer membrane. The mechanism of LptD/E lateral translocation of LPS is different from that of other lateral transport barrels[22-25], as LPS must be delivered specifically to the outer membrane external leaflet. The barrel of LptD is essentially a closed circle, despite the opening observed between strands β1 and β26, as the nonconsecutive disulfide

bonds link the N-terminus of LptD and the periplasmic loop between strands β24 and β25 of LptD (Fig. 3c). The lateral opening between the strands β1 and β26 and the disulfide bonds would appear to ensure that LPS must enter the barrel and insert correctly into the outer leaflet of the outer membrane rather than the inner leaflet of the outer membrane.

Methods Summary

The *LptD* and *LptE* genes of *S. typhimurium* strain LT2 were cloned into pET-28b(+) and pACYCDuet-1, respectively, and the LptD/E complex was over-expressed using the two plasmids in *E.* coli C43(DE3). The LptD/E complex was purified using nickel affinity and gel filtration chromatography. The four wavelength MAD datasets were collected on beamline I24 at Diamond Light Source using selenomethionine-labeled crystals. For functional assays of LptE and LptD, *E. coli* strain AM689, *E.coli* strain AM661 and pACYCDuet-1 plasmid were used. In order to test the LptD and LptE expression levels, hexa-Histag was introduced into the LptD after the signal peptide cleavage site and the C-terminus of LptE, and the Western blots were carried out using His-tag antibody. All molecular dynamics simulations of LptDE complex were performed using GROMACS v4.6.4.

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Supplementary information is available

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Author contributions CJD and WJW conceived and designed the experiments. HD, QJX, CH expressed, purified and crystallized the LptD/E complex, and YHG and ZSW did the mutagenesis and the functional assays. CJD, NP, HD and WJW undertook data collection and structure determination. QJX, ZSW and YZZ generated the constructs for the protein expression and PJS did the molecular dynamics simulation. CJD, HD, WJW and NP wrote the manuscript.

Author information The atomic coordinate and the structure factor of the LptD/E is deposited at the Protein Data Bank under access code 4N4R. The authors declare no financial competing interests. Correspondence and requests for materials should be addressed to CJD (c.dong@uea.ac.uk) or WJW (wenjian166@googlemail.com).

Figure legends

Figure 1 | Crystal structure of the LptD/E complex. The LptD barrel is shown in rainbow, the N-terminal strands coloured in blue, the C-terminal strands in red, and LptE in purple. E, P and OM represent extracellular side, periplasmic side and outer membrane, respectively. a, Schematic representation of LPS. LPS contains three components: hydrophobic lipid A, hydrophilic core oligosaccharide and a polymeric chain of O-antigen (n ranges from 4 - 40). b, Cartoon representation of the LptD/E complex. The C-terminal domain of LptD forms the largest barrel to date, consisting of 26 anti-parallel β strands with 13 extracellular loops. LptE is located inside the barrel. c, d, side view of the LptD/E complex in the outer membrane. The 13 extracellular loops and LptE seal the barrel pore, and LptE is at one side of the barrel along the barrel's y axis. c rotates 180° along the y axis to d. e, top view of the LptD/E complex. Lp4 and Lp8 are located in the interior of the barrel and other loops at the surface of the barrel pore. f, the bottom view of the LptD/E complex. LptE is located inside the LptD barrel and interacts with LptD. e rotates 180° along x axis to f.

Figure 2 | The LptE and LptD interaction. LptD and LptE are coloured the same as in Figure 1. a, The cartoon representation of the LptE structure from *S. typhimurium*. b, The LptE structure, in complex, superimposes well with the LptE structure from *Shewanella oneidensis* (rmsd of 1.68 over 131 Cα). LptE of *S. oneidensis* is shown in cyan. c, Schematic representation of LptD and LptE interactions. d, LptE interaction with Lp8 of LptD. e, LptE interaction with residues on Lp4, Y680 and the C-terminal

residues T771-M786 of LptD. Residues A87-T95 of LptE interact with the residues on Lp4 and C-terminal residues of LptD. f. LptE residues W21-L23 interact with hydrophobic residues at the outer side of the LptD barrel and a zinc ion mediates the LptD/E interaction. The Zn ion is from the crystallization buffer. g, Truncation and deletion mutations of interaction residues impair cell growths in LB medium with 0.5% SDS and 1 mM EDTA. 1, 2, 3, 4 and 5 are the AM689 cells with wild-type *lptE*, the empty pACYCDuet-1, $lptE_{\Delta W21\text{-}L23}$, $lptE_{\Delta A87\text{-}T95}$, $lptE_{\Delta T170\text{-}N196}$, respectively, while 6, 7, 8 are the AM661 cells with wild-type *lptD*, the empty pACYCDuet-1, and $lptD_{\Delta T771\text{-}M786}$. h, The protein expression levels of the wild-type LptE and the deletions are comparable. i, The protein expression levels of the wild-type and the deletion of LptD are similar.

Figure 3 | The unique barrel and pore of the LptD/E complex. The colour of the cartoon representation is identical to Figure 1. In the electrostatic surface potential map, negatively charged residues are shown in red and positively charged residues in blue. E, P and OM indicate extracellular side, periplasmic side and outer membrane, respectively. a, top view of the electrostatic surface potential map of the LptD pore. The yellow dotted line shows the pore, which is covered by extracellular loops with both charged and hydrophobic residues. LptE blocks a hole at one side of the pore. b, bottom view of electrostatic surface potential map of the barrel. The inside of the barrel is very hydrophilic, with LptD C-terminal residues overlapping the surface of the lumen and narrowing the barrel. c, the twisted barrel. The title angle of the N-terminal strands is 30° and the title angle of the C-terminal strands is 67°. Strands β1 and β26 are separated at the periplasmic side, which is highlighted by a yellow dotted circle. The positions of C726 and C727 for disulfide bond formations shown in a blue dotted circle. d, The interaction between strands β1 and β26. Only five hydrogen

bonds are formed between the two strands. e, the side view of the LptD barrel, which shows the hydrophobic belt of the barrel exterior. The yellow dotted circle shows the separation between strands β1 and β26. f, double cysteine mutation that potentially locks β1 and β26 causes the death of the *E. coli*. 1, 2, 3, 4, 5, 6, 7 and 8 represent AM661 cells with the wild-type *lptD*, the empty pACYCDuet-1, *lptD* double mutation N232C/N757C, N232C, N757C, ΔQ722-A729, N232D/N757R and N232Y/N757H, respectively. g, The LptD expression levels of the wild-type and mutants are comparable.

Figure 4 | The proposed mechanism of LPS transport. 1, The newly synthesized LptD has not formed the disulfide bonds and the N-terminal domain of LptD is flexible. 2, The LptD forms the disulfide bonds (red lines), resulting in the conformational change of the N-terminal domain of LptD. The disulfide bond formation helps a lateral opening between strands β1 and β26. 3, The N-terminal domain of LptD interacts with LptA, forming the seven protein transenvelope complex. The LPS is highly negatively charged and the electrostatic repulsion between the LPS probably makes the LPS molecules separately in the inner membrane in the periplasm. By using the ATP, the LPS molecules are extracted from the inner membrane and passed them to LptC, LptA and LptD/E complex, triggering the opening of the LptD pore channel and the lateral gate simultaneously. The O-antigen of LPS pass through the LptD pore channel and the lipid A of LPS is finally inserted into the outer leaflet of outer membrane via the lateral gate between strands β1 and β26 of LptD. The divalent cations are rich at the outer leaflet of outer membrane and the divalent cations are well known to bring the neighboring LPS molecules together. The electrostatic interaction would drive the newly arrived LPS molecules to insert the lipid A precisely at the outer leaflet of outer membrane.

Hundreds and thousands of LPS molecules are delivered to the outer leaflet to form the outer membrane through this mechanism.