

Dynamic basis of lipopolysaccharide export by LptB₂FGC

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Abstract

Lipopolysaccharides (LPS) confer resistance against harsh conditions, including antibiotics, in Gram-negative bacteria. The lipopolysaccharide transport (Lpt) complex, consisting of seven proteins (A-G), exports LPS across the cellular envelope. LptB₂FG forms an ATP-binding cassette transporter that transfers LPS to LptC. How LptB₂FG couples ATP binding and hydrolysis with LPS transport to LptC remains unclear. We observed the conformational heterogeneity of LptB₂FG and LptB₂FGC in micelles and/or proteoliposomes using pulsed dipolar electron spin resonance spectroscopy. Additionally, we monitored LPS binding and release using laser-induced liquid bead ion desorption mass spectrometry. The β-jellyroll domain of LptF stably interacts with the LptG and LptC β-jellyrolls in both the apo and vanadate-trapped states. ATP binding at the cytoplasmic side is allosterically coupled to the selective opening of the periplasmic LptF β-jellyroll domain. In LptB₂FG, ATP binding closes the nucleotide binding domains, causing a collapse of the first lateral gate as observed in structures. However, the second lateral gate, which forms the putative entry site for LPS, exhibits a heterogeneous conformation. LptC binding limits the flexibility of this gate to two conformations, likely representing the helix of LptC as either released from or inserted into the transmembrane domains. Our results reveal the regulation of the LPS entry gate through the dynamic behavior of the LptC transmembrane helix, while its β-jellyroll domain is anchored in the periplasm. This, combined with long-range ATP-dependent allosteric gating of the LptF β-jellyroll domain, may ensure efficient and unidirectional transport of LPS across the periplasm.

eLife assessment

This study provides an **important** advance in the molecular understanding of the lipopolysaccharide export mechanism and machinery in bacteria. By using advanced spectroscopy approaches, the experiments provide **convincing** biophysical support for the dynamic behavior of the multisubunit Lpt transport system. This work has implications for understanding bacterial cell envelope biogenesis and developing drugs that target Gram negative pathogens.

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Introduction

Antibiotic resistance poses a critical threat to global health, necessitating a comprehensive understanding of bacterial defence mechanisms. The outer membrane (OM), a crucial component of Gram-negative bacteria, serves as an initial line of defence against antibiotics¹. Lipopolysaccharides (LPS) constitute a major component of the OM. LPS is a glycolipid consisting of lipid A, core oligosaccharides and an O-antigen². The LPS transport (Lpt) system plays a central role in the biogenesis of OM, contributing significantly to its impermeability and resilience against environmental stress and host defenses³⁻⁸. The pathways for LPS synthesis, transport and regulation offers potential targets for novel antibiotics⁹⁻¹³. Comprising of seven essential components LptA-G (**Figure 1A**), the system orchestrates unidirectional translocation of LPS from the inner membrane (IM) to the OM^{6,14}. The LptB₂FG complex forms an ATP-binding cassette (ABC) transporter in the IM^{4,15-17}. The LptB subunits constitute the nucleotide-binding domains (NBD)¹⁸ and interact in a head-to-tail manner to create two nucleotide binding sites (NBSs, for ATP or ADP). The F and G subunits together form the transmembrane domains (TMDs) to create the LPS binding pocket¹⁹⁻²¹. The pseudo two-fold symmetry of the TMDs creates two lateral gates on either side of the TMDs. LptC connects LptF-LptG^{20,22,23} with LptA²⁴ to transfer LPS through the trans-periplasmic bridge towards LptDE²⁵⁻²⁸.

Previously, LptB₂FG apo structures revealed open NBDs with bound LPS inside the TMDs (**Figure 1B-C**)^{20,22,29}. Upon vanadate trapping, the NBDs dimerize causing a collapse of the LPS binding pocket, which has been suggested as a post-translocation state (**Figure 1D-E**)^{20,23}. Presence of LptC enlarged the cavity leading to a weaker interaction with LPS (**Figure S1A-B**)^{20,22,23}. In this conformation, the transmembrane (TM) helix of LptC weakly interacts with LptG, but forms extensive hydrophobic interactions with TM5 of LptF. The periplasmic β -jellyroll domains of LptF and LptG are very flexible leading to a reduced resolution. LptB₂FGC structures in the AMP-PNP or vanadate-trapped state did not reveal the transmembrane helix of LptC (TM-LptC), thereby revealing a nearly identical conformation of NBDs and TMDs as with the LptB₂FG structures^{20,23}. Also, the β -jellyroll domains were barely resolved (**Figure S1C-D**).

It was proposed that upon ATP binding, TM-LptC moves away from the LptG-TM1 – LptF-TM5 interface. This would lead to a collapse of the binding pocket and expulsion of LPS to the β -jellyroll domain of LptF. However, several details of this mechanism such as when and how TM-LptC dissociates and in what way that is transmitted to the interaction and dynamics of the β -jellyroll domains remain poorly understood. Likely, many intermediate states might exist between ATP binding and LPS translocation and the relevance of the available structures in a native-like lipid environment is unknown. Altogether, an integrated characterization of the intermediate structures and underlying dynamics is required to elucidate the functional mechanism. Here we characterized the conformational heterogeneity of LptB₂FG and LptB₂FGC using extensive pulsed dipolar electron spin resonance (ESR) spectroscopy experiments, which permits observations in micelles, lipid bilayers or even in the native environments for certain cases³⁰⁻⁴¹. These observations were further correlated with results obtained using laser-induced liquid bead ion desorption mass spectrometry (LILBID-MS)^{42,43}. Our results provide novel insights into the allosteric regulation of the structure and dynamics of the TMDs and the β -jellyroll domains during ATP binding and hydrolysis, which altogether drive LPS translocation.

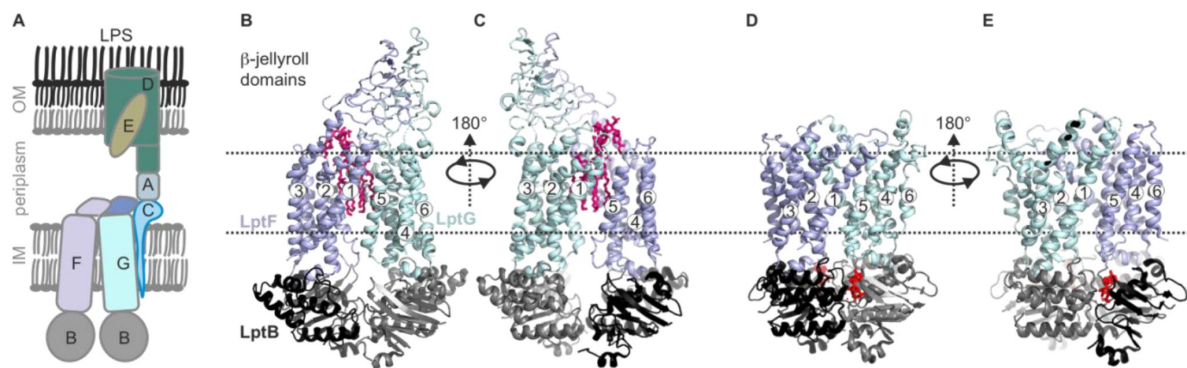


Figure 1.

Lipopolysaccharide transport (Lpt) system.

(A) the Lpt system consists of the LptB₂FG-C complex located in the inner membrane (IM). Periplasmic LptA connects this complex with LptDE, which is located in the outer membrane (OM). (B, C) Structures of LptB₂FG in the LPS-bound state (PDB ID: 6MHU) or (D, E) in the vanadate-trapped state (PDB ID: 6MHZ) are shown. In the vanadate-trapped structure, the cavity is collapsed with no space for LPS and the β -jellyroll domains are not resolved. The LPS molecule (magenta) and nucleotides (red) are highlighted.

Results

Conformational heterogeneity of the NBDs in LptB₂FG in micelles and liposomes

Initially, we characterized the dynamics of LptB₂FG in both DDM micelles and proteoliposomes (PLS) using pulsed electron-electron double resonance (PELDOR or DEER) spectroscopy.^{44,45} This technique has been used for investigating several Type I, type II, type III and type IV ABC transporters.^{31,38,46,52} LptB₂FG belongs to the type VI class of ABC transporter. For this purpose, we engineered spin pairs along the NBDs, TMDs and the β -jellyroll domains (**Figure 2-6**, **Figure S2**). Following reaction with a cysteine, the (1-Oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate (MTSL) label creates the side chain denoted as R1.⁵³ The functionality of the spin labelled variants after size-exclusion chromatography was confirmed using ATP-ase activity assay (**Figure S3-S4**). All the cysteine variants could be labelled with high efficiency (**Figure S5** and **Table S1**). We investigated LptB₂FG under several conditions including the apo-state or after incubating with ATP-EDTA (EDTA to chelate divalent metal ions and to inhibit any residual ATPase activity, denoted as ATP), ATP-Mg²⁺-VO³⁻ (vanadate-trapped transition state) or ADP-Mg²⁺ (post-hydrolysis state).

In agreement with the apo structure, the NBDs displayed a mean interspin distance centred around 5 nm in micelles when observed at position B_M134R1 (**Figure 2A-D**). This distance decreased below 4 nm in the vanadate-trapped state. With ATP a mixed population was observed with the major peak corresponding to the closed conformation. Thus, Mg²⁺ ions are not necessary for the closure of the NBDs. With ADP-Mg²⁺ the NBDs do not close, though the overall flexibility is somewhat increased (as gauged from the width of the distribution). We performed additional experiments after reconstitution into PLS (**Figure 2F-G**). Overall, the data in the apo-state revealed a similar mean distance, yet with a larger width suggesting an increased dynamics. As observed in micelles, vanadate-trapping closed the NBDs in PLS as well. Overall, the nucleotide-induced closure as observed in micelles (and the structures) is maintained in the native-like lipid bilayers for the NBDs.

Conformational heterogeneity of the lateral gate-1 of LptB₂FG in micelles and PLS

We introduced two spin pairs to independently monitor the lateral gates of the TMDs. The lateral gate formed by LptF-TM1 – LptG-TM5 and LptG-TM1 – LptF-TM5 were monitored using F_A45R1 – G_I325R1 and F_L325R1 – G_A52R1 pairs, respectively. For the apo-state in micelles, the distances for the lateral gate-1 are similar to the corresponding simulation on the LPS-bound structure (**Figure 3A**). Vanadate-trapping resulted in an increase of the distances in agreement with the simulation. Though the central cavity collapses, this increased distance results from a coordinated movement of LptF-TM1 towards the cavity accompanied with an outward shift of LptG-TM5 (**Figure 3E** and **Figure S1-S2**). Addition of ATP resulted in an equilibrium between the two conformations. Altogether, the PELDOR data validate a LPS binding competent conformation in the apo- and ADP-Mg²⁺ states accompanied with a collapse of the gate in the ATP and vanadate-trapped post-hydrolytic state. Interestingly, for the apo state in PLS, this gate exhibited a broader distribution spanning the range corresponding to both structures (**Figure 3F-G**). In the vanadate-trapped state the overall distribution got narrowed (the minor peak at a longer distance is not interpreted due to a larger uncertainty). However, the overall spread of the distribution remained similar, suggesting a more flexible conformation of this gate in PLS, which is minimally affected by ATP binding.

Figure 2.

DEER/PELDOR data for the NBDs of LptB₂FG in micelles and PLS.

(A-D, F-G) Primary data overlaid with the fits obtained using the DeerLab⁵⁴ program (A-D in micelles) or ComparativeDeerAnalyzer^{54,55} (F-G in PLS) are shown in the left panels. The obtained distance distributions with a 95% confidence interval are shown on the right. Simulations for the open (PDB ID: 6MHU) or closed (PDB ID: 6MHZ) structures are overlaid with the apo or vanadate-trapped distances, respectively (in dotted orange line). (E) The spin labelled positions (as sphere) and the loops carrying them are highlighted (in red) on the open and closed structures. The PLS samples were analysed using ComparativeDeerAnalyzer to account for the entire uncertainty including the partial dimensionality for spin distribution over the membrane surface. The colour code for the distance distribution (F-G) relates the reliability for different features of the probability distribution with respect to the length of the observed dipolar evolution time. In the green zone, shape, width, and the mean distance are accurate. In the yellow zone, width and the mean, and in the orange zone, the mean distance are reliable. The arrow indicates the conformational change from the apo-state upon nucleotide binding.

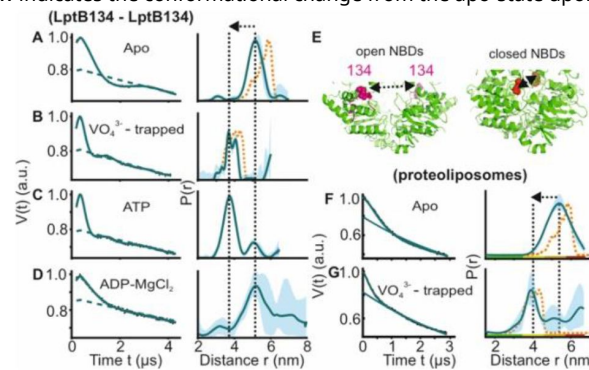
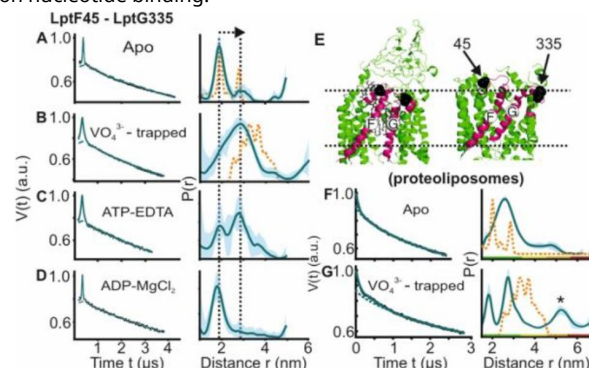


Figure 3.

DEER/PELDOR data for the lateral gate-1 between LptF-TM1 – LptG-TM5 of LptB₂FG in micelles and PLS.

Primary data overlaid with the fits obtained using the DeerLab program (A-D in micelles) or ComparativeDeerAnalyzer (F-G in PLS) are shown in the left panels. The obtained distance distributions with a 95% confidence interval are shown on the right. (E) The spin labelled positions are highlighted on the open (PDB ID: 6MHU) and closed (PDB ID: 6MHZ) structures and the corresponding simulations are overlaid with the apo or vanadate-trapped distances, respectively (in dotted orange line). The distance peak indicated with an asterisk in G is not interpreted for the reason of a larger uncertainty. The colour code for the distance distribution (F-G) corresponds to the description as given in [Figure 2](#). The arrow indicates the conformational change from the apo-state upon nucleotide binding.



The putative lateral gate-2 for LPS entry exhibits a large conformational heterogeneity

Interestingly, the lateral gate-2 between LptG-TM1 – LptF-TM5 revealed a more dynamic behaviour in micelles (**Figure 4A-D**). In the apo state, it has a broad conformational distribution spanning the LPS bound (open) and vanadate-trapped (closed) structures (corresponding simulations are shown in orange lines). In the vanadate-trapped and ATP samples, the major population is centred at 2 nm (which is closer to the simulation on the vanadate-trapped structure). The decreased interspin distances arise from a collapse of the cavity upon vanadate-trapping (**Figure 4E** and **Figure S1-S2**). ADP-Mg²⁺ also gave a broad distribution comparable to the apo-state. Thus, in the apo-state this gate appears to exist in an equilibrium between the two conformations as observed from the corresponding structures. ATP binding or vanadate-trapping shifts the equilibrium towards the collapsed conformation. In PLS as well, the apo state revealed a broad distribution validating the heterogeneity in the membrane environment (**Figure 4F-G**). Vanadate-trapping somewhat narrowed the distribution with (only) a fraction of the distances overlaying with the simulation for the corresponding structure (and with a more pronounced heterogeneity as compared with the micellar sample, **Figure 4B**). Position A52 on LptG is located at the beginning of the neighboring TM2. Position L325 is located on the short loop between TM5 and TM6 in LptF. This loop is resolved with clear density and has a similar orientation in the apo and vanadate-trapped structures with minimum deviation (**Figure 4E**, **S6**). Further, the observed heterogeneity is distinctly modulated upon LptC binding (**Figure 6D-E**), suggesting that the internal flexibility around the labelled sites might have the least contribution to the broad distribution we experimentally observed. Confirming these observations, the room temperature continuous wave ESR spectra revealed the least flexibility for this spin pair (**Figure S5**, **S7**). Comparing this heterogeneity from the PELDOR data (**Figure 4**) with the corresponding simulations (in orange, **Figure 4A-B**), it appears that the structures captured two of the states from the broad conformational space. This gate is suggested to be the entry point for LPS²² and the enhanced dynamics we observed might be required for efficient interaction with LPS and LptC (please see later sections). Supporting these observations, LptG-TM1 and LptF-TM5 helices interact rather weak in the structures, in particular in the LPS-bound state (**Figure 1B-C**, and **Figure S1-S2**).

Conformational dynamics of the β -jellyroll domains

We further investigated the interaction between the β -jellyroll domains in LptB₂FG. Distances between positions F_S186R1 and G_V209R1 located on these domains revealed a major peak in agreement with simulation on the LPS-bound structure (**Figure 5A-B**) and a previous study⁵⁶. The overall spread of the distribution remained rather similar in the vanadate-trapped state revealing a stable interaction. We also probed the internal flexibility of LptF β -jellyroll using F_S156R1 – I234R1 and F_S186R1 – I234R1 pairs, which are located on the putative entry gate loops for LPS²² (**Figure 5C, E-F**). The LptF β -jellyroll in the apo-state showed a broad distribution, but with a major population staying in the closed conformation as observed in the structure (**Figure 5E, F**). Interestingly, in the vanadate-trapped state, the overall distribution shifted towards longer distances revealing an opening of the LPS entry gate. Altogether, these observations reveal a long-range allosteric coupling between nucleotide binding at the NBDs in the cytoplasm and opening of the LPS entry gate of LptF β -jellyroll in the periplasm. Such a tight coupling might prime this domain to receive LPS once it is released from the cavity inside the TMDs. A strikingly different response was observed for the β -jellyroll domain of LptG. The equivalent spin pairs G_V209R1 – G_L234R1 and G_L152R1 – V209R1 revealed a considerably enhanced flexibility independent of ATP (**Figure 5D, G-H**) and revealing no such allosteric coupling with the NBDs.

Figure 4.

DEER/PELDOR data for the lateral gate-2 between LptF-TM5 – LptG-TM1 of LptB₂FG in micelles and PLS.

Primary data overlaid with the fits obtained using the DeerLab program (A-D in micelles) and DeerNet (F) or ComparativeDeerAnalyzer (G) in PLS are shown in the left panels. The obtained distance distributions with a 95% confidence interval are shown on the right. Due to a larger uncertainty arising from the broad distribution and the limited time-window, output from DeerNet is shown in F. (E) The spin labelled positions are highlighted on the open (PDB ID: 6MHU) and closed (PDB ID: 6MHZ) structures and the corresponding simulations are overlaid with the apo or vanadate-trapped distances, respectively (in dotted orange line). The colour code for the distance distribution (F-G) corresponds to the description as given in [Figure 2](#). The arrow indicates the conformational change from the apo-state upon nucleotide binding.

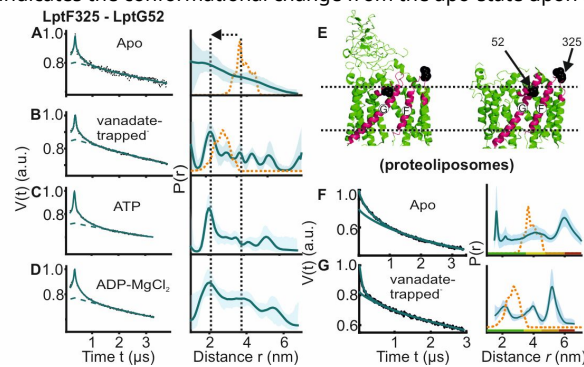
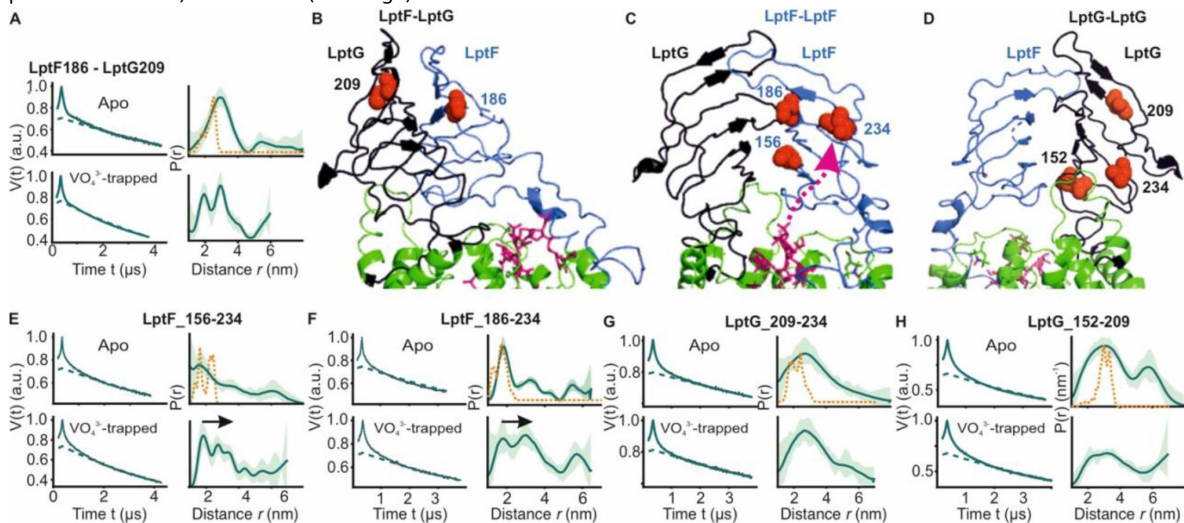


Figure 5.

DEER/PELDOR data for β -jellyroll domains of LptB₂FG.

(A) Primary data for LptF-LptG β -jellyrolls overlaid with the fits obtained using the DeerLab⁵⁴ program are shown in the left panels. The obtained distance distributions with a 95% confidence interval are shown on the right. (B-D) The spin labelled positions are highlighted on the LptB₂FG apo structure and the bound LPS is shown in stick representation (PDB ID: 6MHU). (E, F) Primary data overlaid with the fits and the corresponding distance distributions (right) from PELDOR experiments within the β -jellyroll domain of LptF or LptG (G, H). Corresponding simulations (where structures are available having the positions resolved) are overlaid (in orange).



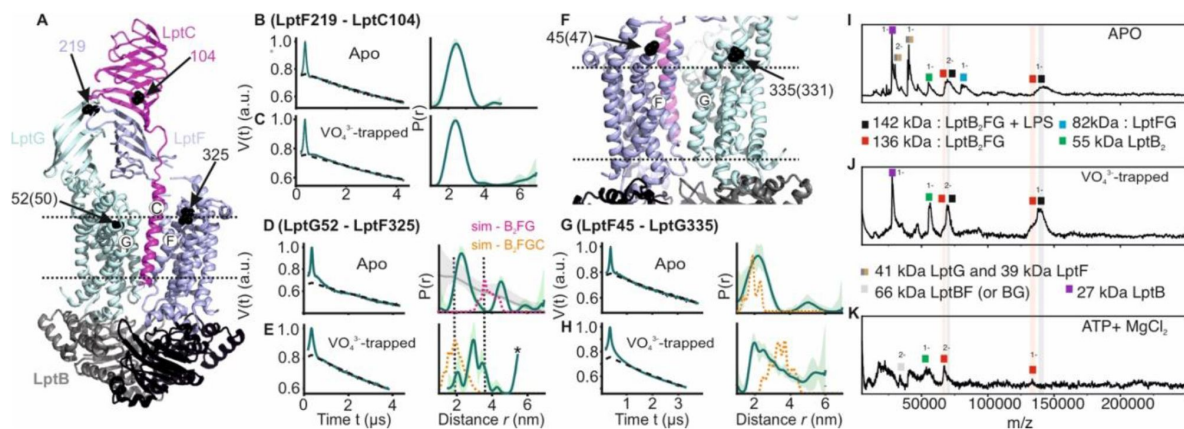


Figure 6.

DEER/PELDOR data for the lateral gates of LptB₂FGC and LILBID-MS.

(A) The LptB₂FGC structure (PDB ID: 6MJF) from *Vibrio cholerae*. The helices from LptF and LptG forming the lateral gate-2 is shown and the positions analogous to those we investigated in the *E. coli* structure are highlighted (in sphere representation and indicated with an arrow, when the amino acid number is different, it is indicated inside the bracket). Similarly, the lateral gate-1 is highlighted in panel F. (B-E, G-H) Primary PELDOR data overlaid with the fits obtained using the DeerLab⁵⁴ are shown in the left panels. The obtained distance distributions with a 95% confidence interval are shown on the right. The corresponding distances for LptB₂G is overlaid for a comparison (in grey for the apo state) in panel D and asterisk in panel E indicate unreliable distances. Simulation on the LptB₂FGC apo structure (in orange) is overlaid. In panel D, corresponding distances for LptB₂FG is overlaid (in grey) and the simulation for LptB₂FG structure is shown (in magenta) as position L325 is not resolved in the *E. coli* LptB₂FGC structure. (I-K) LILBID-MS data for detergent solubilized LptB₂FG in different states as indicated.

Conformational heterogeneity of the the β -jellyroll domains and lateral gates in LptB₂FGC

Next, we investigated how LptC modulates dynamics of the lateral gates and the β -jellyroll domains in LptB₂FG. Measurements between the β -jellyroll domains of LptF and LptC (F_L219R1 – C_T104R1) revealed a narrow distance distribution and hence a stable interaction between them in both apo and vanadate-trapped states (**Figure 6A-C**). At the lateral gate-1 (F_A45R1 – G_I335R1, **Figure 6F** and **G-H**), the distance in the apo state is similar to the simulation on the structure. In the vanadate trapped state, the spread of the distance distribution is increased, spanning the range of the simulations corresponding to both apo and vanadate-trapped structures (in the latter structure, TM-LptC is absent at the gate leading to a collapse of the cavity and an identical conformation with LptB₂FG, though the distances increase due to an outward movement of LptG-TM5, see **Figure 3B,G** and **Figures S1-S2**). Distances corresponding to the apo state are present possibly due to an incomplete vanadate-trapping (or an equilibrium between the two conformations, which is rather unlikely) for this sample. Overall, the results support TM-LptC dissociation in the vanadate-trapped state as observed in the structure.

A distinct response is observed for the lateral gate-2 (F_L325R1 – G_A52R1, **Figure 6A** and **D-E**). LptC binding reduced the overall flexibility of this gate into two defined distance peaks as compared to LptB₂FG alone (**Figure 4A** vs. **6D**). LptC binding is shown to increase the separation between these helices (**Figure S1A**). The major distance peak (centred around 2 nm) is shorter than the simulation on the LptB₂FG apo structure (in magenta, **Figure 6D**, top panel), which therefore likely represents a conformation in which TM-LptC is released. The second peak corresponds to a larger separation between these helices, possibly representing the structure having the TM-LptC inserted at the gate (as position L325 is not resolved in the *E. coli* structure, corresponding simulation is not presented). The first conformation appears to be more favoured under the experimental conditions. In the vanadate-trapped state, the second peak disappeared and the overall distribution centred towards the TM-LptC released (LptB₂FG) conformation (**Figure 6E**). This is in line with the lack of density for TM-LptC in the vanadate-trapped LptB₂FGC structure. The experimental distribution is longer than the corresponding simulation (orange line in **Figure 6E**), suggesting a somewhat farther separation between these helices.

We further probed LPS release in LptB₂FG using LILBID-MS (**Figure 6I-K**). In the apo and vanadate-trapped states, the majority of the observed complex peaks represent LPS bound to LptB₂FG, while some LPS free LptB₂FG can as well be seen (indicated in grey and red shades, respectively for both 1- and 2-charge states). Thus, LPS is co-purified with LptB₂FG in micelles. Presence of LPS-bound LptB₂FG in the vanadate-trapped state is not unexpected as the PELDOR data revealed a heterogenous conformation for the LPS entry gate other than the collapsed conformation (**Figure 4B, G**). However, under the hydrolysing conditions, LPS is completely released from LptB₂FG.

Discussion

LPS transport by LptB₂FG has remained elusive for many reasons. LptB₂FG and LptB₂FGC structures revealed a similar structure in the vanadate-trapped state leaving no trace for TM-LptC and the β -jellyroll domains were not resolved (**Figure S1D**). Available structures possess either AMP-PNP or ADP-VO₄³⁻ as nucleotides (both in a closed conformation of the TMDs and NBDs), thereby limiting details on how ATP binding and hydrolysis are coupled to the conformational changes and LPS transport.

For the NBDs and the two lateral gates of LptB₂FG, PELDOR data validated the conformational changes in micelles as observed from the structures. Upon ATP binding and/or vanadate-trapping, the NBDs and the two lateral gates moved in a coordinated manner to collapse the LPS binding pocket (**Figures 2**–**4**). However, the lateral gate-2 forming the LPS entry site (TM1G–TM5F monitored by F_L325 – G_A52, **Figure 4**, **S1** and **S2**) exhibits a flexible conformation, which might facilitate interaction with LPS and/or LptC. In agreement, LptC binding restricted the overall flexibility of this gate into two distinct conformations corresponding to a collapsed and an open conformation in which TM-LptC is likely inserted into the TMDs (**Figure 6D**). The minor peak at longer distances might correspond to a conformation in which TM-LptC and LPS can enter the TMDs while the β -jellyroll domains of LptC and LptF interact. The minimal interaction between the corresponding helices (**Figure S1**–**S2**) in the LptB₂FG structures suggest that the flexibility is inbuilt and might have an important role for the function. The PLS environment modulates the observed conformation in LptB₂FG. The lateral gate-1 has a broader distribution in PLS, which is minimally affected by vanadate-trapping (**Figure 3F–G**). Also, the heterogeneity for the lateral gate-2 is more pronounced in PLS. The liposomes are made from *E. coli* polar lipid extract. In the polar lipid extract, phosphatidylethanolamine is the predominant lipid component with minor amounts of phosphatidylglycerol and cardiolipin. Thus, the differences in the heterogeneity we observed in proteoliposomes might not be due to the presence of LPS. Overall, the observations in PLS are qualitatively similar to the micellar sample (also see **Figure S7** for the continuous wave (cw) ESR spectra) and further experiments are required to clarify how the membrane would influence LptB₂FGC conformation.

In LptB₂FG, the two β -jellyroll domains stably interact in the apo and vanadate-trapped conditions (**Figure 5**). Interestingly, binding of nucleotides is allosterically coupled to a selective opening of LptF β -jellyroll with little effect on the LptG β -jellyroll (**Figure 5E–H**). This opening was shown to be essential for cell growth and it was suggested that movement of LPS, but not ATP binding or hydrolysis pushes the gate open²². However, our results confirm that the gate opening is allosterically regulated through ATP binding and/or hydrolysis. This β -jellyroll starts from TM3 and ends on TM4. TM3 is connected to the coupling helix and TM4 is connected to the cytoplasmic loop-2, which harbours the conserved R292 directly interacting with the nucleotide²⁰. It is likely that one or both of these motifs are involved in the allosteric communication. Notably, the LptG β -jellyroll domain exhibits significant internal flexibility (**Figure 5G–H**). Biochemical studies showed that conserved residues in this domain are essential for cell growth²⁰. Though there is little evidence for a direct role of this domain in LPS binding, the observed dynamics might be important for efficient transport. Similarly, in LptB₂FG-C, the β -jellyrolls of LptC and LptF interact both in both apo and vanadate-trapped states (**Figure 6A–C**), which altogether might form a continuous pathway for LPS transfer towards LptA. The β -jellyrolls were not fully resolved in the vanadate-trapped structure of LptB₂FG (**Figure 1**) and LptB₂FGC²³, revealing a considerable flexibility. The exact role for this enhanced dynamics is unclear and it may somehow facilitate LPS release. The narrow distance distributions we observed reveal that these domains might move as rigid bodies while sampling a broad conformational space.

The LILBID-MS data show that LPS is released upon ATP hydrolysis by LptB₂FG (**Figure 6I–K**, **Figure 7 steps 1–2**). Thus, LptC is required for a productive transport cycle in the wild-type protein^{24,57,58}. Our observations suggest that the TM-LptC dynamically associates and dissociates at the lateral gate-2, even in the apo-state while its β -jellyroll domain is firmly bound to LptF β -jellyroll (**Figure 7, step 3**). Such a dynamic behaviour of TM-LptC has previously been suggested based on the *Klebsiella pneumoniae* LptB₂FGC structure, which showed density for the short N-terminus segment of TM-LptC⁵⁹. Vanadate trapping induces the TM-LptC released conformation of the lateral gates (**Figure 6D–E** and **G–H**), in agreement with the structures, which also leads to LPS release^{20,22}. Thus, ATP binding/hydrolysis might be coupled to the collapse of the TMDs and expulsion of LPS. Inferring from our observations on LptB₂FG (**Figure 5**), ATP binding might allosterically open the LptF β -jellyroll (**Figure 7, step 4**), which might subsequently close after passing LPS to the LptC β -jellyroll to prevent any backflow²⁰ (**Figure 7,**

step 5 [↗](#)). Through repeating this cycle, LptB₂FG might push LPS along the trans-periplasmic bridge in a unidirectional manner according to the Pez candy dispenser model⁶⁰ [↗](#). The possibility to observe LptB₂FG and LptB₂FGC offers further opportunity to characterize the dynamics of the β -jellyroll domains, LptB₂FG – TM-LptC interaction as well as other intermediate states of the transport cycle even in the native-like lipid bilayers. These experiments, which are beyond the current scope of the study are in progress in our laboratory. As the persistently interacting, yet dynamic LptB₂FGC complex forms the functional LPS transport unit in the inner membrane, a detailed understanding of its dynamics would facilitate development of novel drugs against Gram-negative bacterial pathogens.

Materials and Methods

Expression, purification and spin labeling of LptB₂FG and LptB₂FG-C

The plasmid pETDuet-*lptB-lptFG*, including a C-terminal His-tag on LptB, was used to transform in *E. coli* BL21(DE3) C43 for LptB₂FG expression. For LptB₂FGC a co-expression with the pCDFDuet-1-*lptC* together with pETDuet-*lptB-lptFG* was performed. For bacterial cell growth Luria broth (LB) medium with 100 μ g/mL ampicillin (for LptB₂FG, and additionally 50 μ g/mL streptomycin for LptB₂FGC) was incubated at 37 °C until an optical density of 1.0 at 600 nm. The protein expression was induced with 0.2 mM or 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for LptB₂FG and LptB₂FGC, respectively. After overnight expression at 18 °C the cells were collected by centrifugation. Cell pellets were resuspended in lysis buffer (20 mM HEPES, pH 7.5, 300 mM NaCl) with a homogenizer and lysed by French press with an additional spatula tip of DNase I, lysozyme and 100 μ g/ml PMSF. Cell debris was removed by centrifugation at 12,000xg for 20 min at 4 °C. The membranes were pelleted by ultra-centrifugation at 200,000xg for 2 h at 4 °C. Membranes were flash-frozen in liquid nitrogen and stored at -80 °C. For solubilization lysis buffer with 1% (w/v) DDM was used and after homogenization membranes were incubated for 30 min at 4 °C. The protein solution was ultra-centrifugated at 100,000xg for 35 min at 4°C. The supernatant was incubated with washed Ni Sepharose (High Performance, GE Healthcare) for 45 min at 4 °C and loaded on an empty PD-10 column (GE Healthcare). The column was washed with 15 column volumes of wash buffer containing 20 mM HEPES, pH 7.5, 300 mM NaCl, 0.05% (w/v) DDM, 5% (v/v) glycerol and 40 mM imidazol. The protein bound to the Ni Sepharose was labeled with 2 column volumes of 20 mM HEPES, pH 7.5, 300 mM NaCl, 0.05% (w/v) DDM, 5% (v/v) glycerol and 300 μ M MTSL (1-Oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate for 1.5 h at 4°C. The column was washed with 40 column volumes of the same buffer (without MTSL) and further eluted with 300 mM imidazole and immediately desalted using a PD-10 desalting column (GE Healthcare) into the same buffer. The protein was then concentrated to about 100 μ M (Amicon Ultra-15, PLQK Ultracel-PL Membrane, 50 kDa, Merck, Millipore). After size-exclusion chromatography with a Superdex 200 Increase10/300 GL column (GE Healthcare), the protein fractions were collected and concentrated to ~40 μ M protein. For PELDOR spectroscopy different samples were prepared. For the ATP-EDTA sample, 5 mM ATP and 0.5 mM EDTA were added (5 min, 37 °C). The ADP-MgCl₂ sample contained 5 mM ADP and 5 mM MgCl₂ (1 min, RT). The vanadate-trapped sample was prepared with 5 mM ATP, 5 mM MgCl₂ and 5 mM *ortho*-vanadate and the sample was incubated for 5-10 minutes at 37°C. All the samples were adjusted to the same final protein and DDM concentration (0.05%).

Proteoliposome reconstitution and PELDOR sample preparation

Liposomes (20 mg/mL, 20 mM HEPES, 300 mM NaCl, 10% (v/v) glycerol, pH 7.5) made from *E.coli* polar lipid extract (Avanti) were mixed with 0.14% Triton X-100 and incubated for 30 min at room temperature. Liposomes and protein (in the same buffer additionally containing 5% glycerol and 0,05% (w/v) DDM) were mixed at 1:10 (w/w) ratio to a final lipid concentration of 4 mg/mL in 10

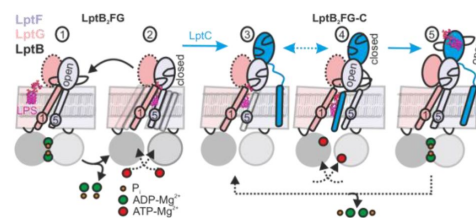


Figure 7.

LPS translocation mechanism.

(1-2) The lateral gate-2 formed by LptG-TM1 and LptF-TM5 in LptB₂FG, which is dynamic in the apo state (2) is shown (see [Figure 4](#)). ATP binding, which leads to hydrolysis (1) collapses the cavity and releases LPS. (3,4) Interaction with LptC limits heterogeneity, creating an equilibrium between TM-LptC inserted and released conformations ([Figure 6D](#)). ATP binding opens the periplasmic gate in LptF β-jellyroll (4) and subsequent closure of the NBDs leads to the collapse of the cavity and LPS transfer. As LPS moves to LptC, LptF β-jellyroll might close accompanied with an opening of LptC β-jellyroll²⁰ (5). Dissociation of ADP-Mg²⁺ will initiate the next cycle according to the Pez mechanism. Dotted line for LptG β-jellyroll indicates enhanced inter-domain dynamics (see [Figure 5G-H](#)).

mL. The protein-lipid suspension was gently incubated for 1 h at room temperature. Afterwards Bio-Beads SM-2 (Bio-Rad) was added and was gently mixed for 30 min at room temperature and moved to 4° C. The Bio-Beads were added three more times for the following incubation periods at 4°C: 1 h, overnight and 2 h. Proteoliposomes were diluted to 2 % (v/v) glycerol in 20 mM HEPES, 300 mM NaCl, pH 7.5 and were pelleted at 200,000xg (4 °C) and resuspended into the same buffer. PELDOR samples were prepared as described above for DDM micelles with an additional freeze-thaw (5x) cycle. Samples were frozen in liquid nitrogen and stored at -80 °C.

ATP assay

The ATP assay was performed according to a protocol modified from Morbach *et al.*⁶¹, 5 µg of purified LptB₂FG or LptB₂FGC were incubated at 37 °C for 8 min in 25 µL reaction volume on a 96-well plate with different ATP concentrations (0, 0.125, 0.25, 0.5, 1, 2, 3 and 5 mM) in 20 mM HEPES pH 7.5, 150 mM NaCl, 0.05 % (w/v) DDM and 5 mM MgCl₂. The reaction was stopped with addition of 150 µL of 20 mM H₂SO₄. A 50 µL working solution [50 ml of ddH₂O, 10 ml of ≥ 95 % H₂SO₄ and 73.4 mg of malachite green chloride, 40 µl of 11 % Tween 20, and 500 of µl 7.5 % (w/v) (NH₄)₆Mo₇O₂₄·4H₂O solution] was added and incubated for 8 min at RT and the absorbance was measured at 620 nm in a microplate reader. The data was analyzed using Origin 2018.

Continuous wave (cw) ESR spectroscopy

Continuous wave ESR spectroscopy measurements were performed on a X-band Bruker EMXnano benchtop spectrometer. A 16 µL of the protein sample was measured in a 0.64 mm diameter micropipette (BRAND, Germany) with 100 kHz modulation frequency, 0.6-2 mW microwave power, 0.15 mT modulation amplitude and 18 mT sweep width.

Pulsed electron-electron double resonance spectroscopy

DEER/PELDOR experiments were conducted on a Bruker Eleksys E580 Q-Band (34 GHz) pulsed ESR spectrometer equipped with an arbitrary waveform generator (SpinJet AWG, Bruker), a 50 W solid-state amplifier, a continuous-flow helium cryostat, and a temperature control system (Oxford Instruments). Measurements were carried out at 50 K using a 10 – 20 µL frozen sample containing 15 – 20% glycerol-d₈ in a 1.6 mm quartz ESR tube (Suprasil, Wilmad LabGlass) with a Bruker EN5107D2 dielectric resonator. The phase memory time (T_M) measurements were performed with a 48 ns $\pi/2-\tau-\pi$ Gaussian pulse sequence with a two-step phase cycling after incrementing τ in 4 ns steps. A dead-time free four-pulse sequence with a 16-step phase cycling ($x[x][x_p]x$) was used for DEER measurements⁶². A 38 ns Gaussian pump pulse (with a full width at half maximum (FWHM) of 16.1 ns) was employed, along with a 48 ns observer pulse (FWHM of 20.4 ns)⁶³. The pump pulse was placed at the maximum of the echo-detected field swept spectrum, and the observer pulses were set 80 MHz lower. Deuterium modulations were averaged by progressively increasing the first interpulse delay by 16 ns over 8 steps. The data analysis was performed with the ComparativeDeerAnalyzer 2.0 (CDA)^{54,55} or the DeerLab program. The distance distributions were simulated on the structures (PDB 6MHU, 6MHX, 6MI7 and 6MI8) using a rotamer library approach as implemented in the MATLAB-based MMM2022.2 software package⁶⁴.

Liquid bead ion desorption mass spectrometry (LILBID-MS)

For analysis by LILBID-MS, samples were buffer exchanged to 20 mM Tris(HCl), 0.05% DDM, pH 7.5. For ATP-Mg²⁺ sample, 2 mM ATP and 0.5 mM MgCl₂ were added prior to the buffer exchange and the sample was incubated for 1 min at room temperature. After dilution to 10 µM protein, 4 µL of the sample were loaded directly into a droplet generator (MD-K-130, Microdrop Technologies GmbH, Germany). This piezo driven device generates droplets of a diameter of around 50 µm at a frequency of 10 Hz. The droplets are then transferred into vacuum, and irradiated by an IR laser working at a wavelength of 2.8 µm having a maximum energy output of 23 mJ per pulse (6 ns). The free ions were accelerated into a homebuilt time of flight analyser by applying an acceleration

voltage in the Wiley McLaren type ion optics. The voltage between the first and the second lens was set to -4.0 kV. At 5 to 25 μ s after irradiation, the first lens was pulsed to -6.6 kV for 370 μ s, while the reflectron worked at -7.2 kV. Detection was conducted by a homebuilt Daly type detector, optimized for high m/z .

Author contributions

M.D. and B.J. designed the experiments, M.D. purified the proteins and performed all biochemical and ESR spectroscopy experiments and analyzed the data together with B.J. T.R. and N.M. performed and analyzed the LILBID-MS experiments. B.J. made the initial draft and finalized the manuscript together with all the authors. B.J. conceived the idea, acquired funding, supervised and administered the project.

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Data Availability

The PELDOR/DEER data are available upon request to B.J.

Supporting Information

Supplementary Figures

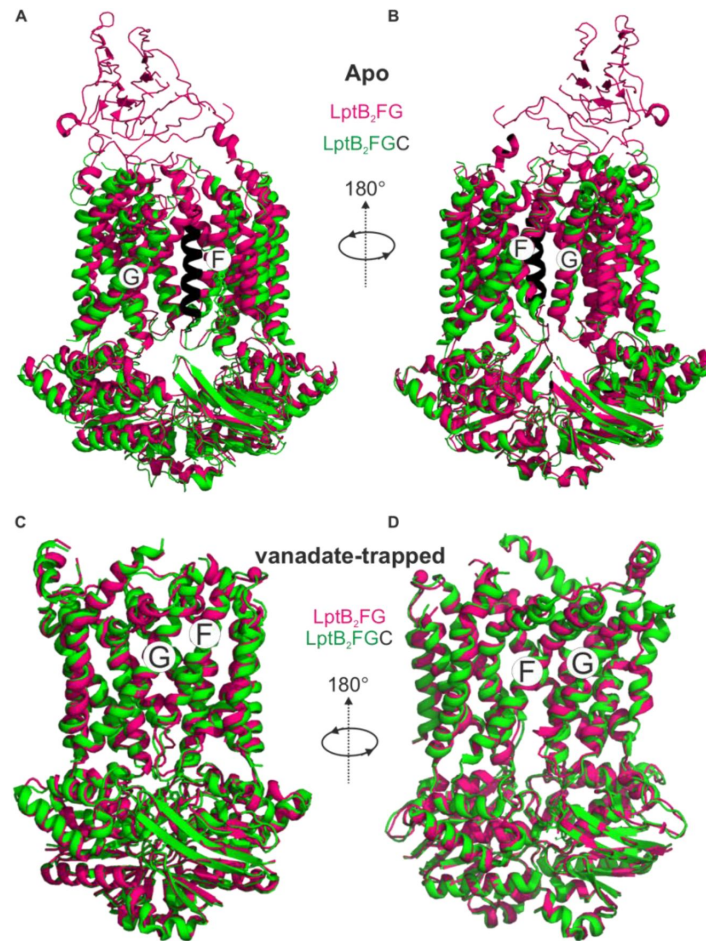


Figure S1.

LptB₂FG and LptB₂FGC structures in the apo and vanadate-trapped states.

(A) *E. coli* LptB₂FG (magenta, PDB ID: 6MHU) and LptB₂FGC (LptB₂FG subunits in green and LptC in black, PDB ID: 6MI7) structures in the apo state are overlaid. The gating helices named as G and F moves apart upon binding LptC. A view from the other lateral gate is shown in (B). (C) *E. coli* LptB₂FG (magenta, PDB ID: 6MHZ) and LptB₂FGC (PDB ID: 6MI8) structures in the vanadate-trapped states are overlaid. The TM-LptC is not resolved in LptB₂FGC and the structures are nearly identical with an overall RMSD of 0.99 Å.

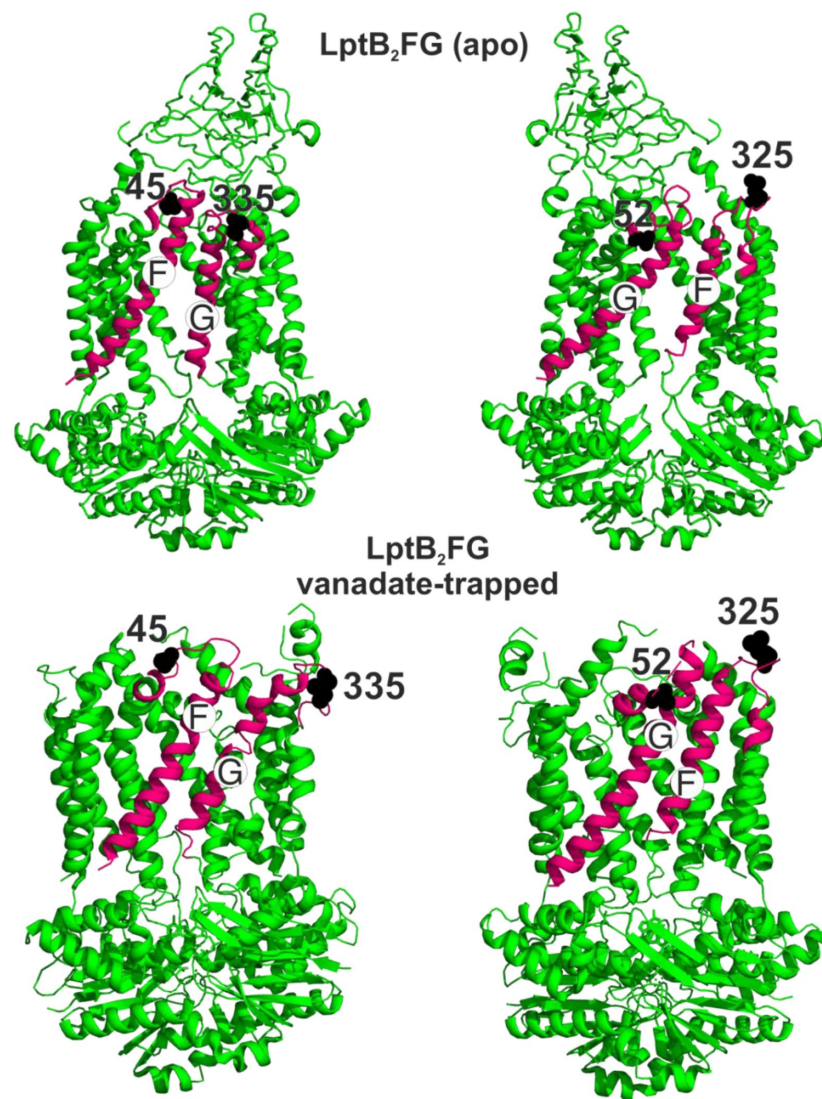


Figure S2.

Spin labeled positions in LptB₂FG.

The two lateral gates (magenta) and the labeled positions (black spheres) are highlighted on the apo (PDB ID: 6MHU) and vanadate-trapped (PDB ID: 6MHZ) LptB₂FG structures (top and bottom, respectively).

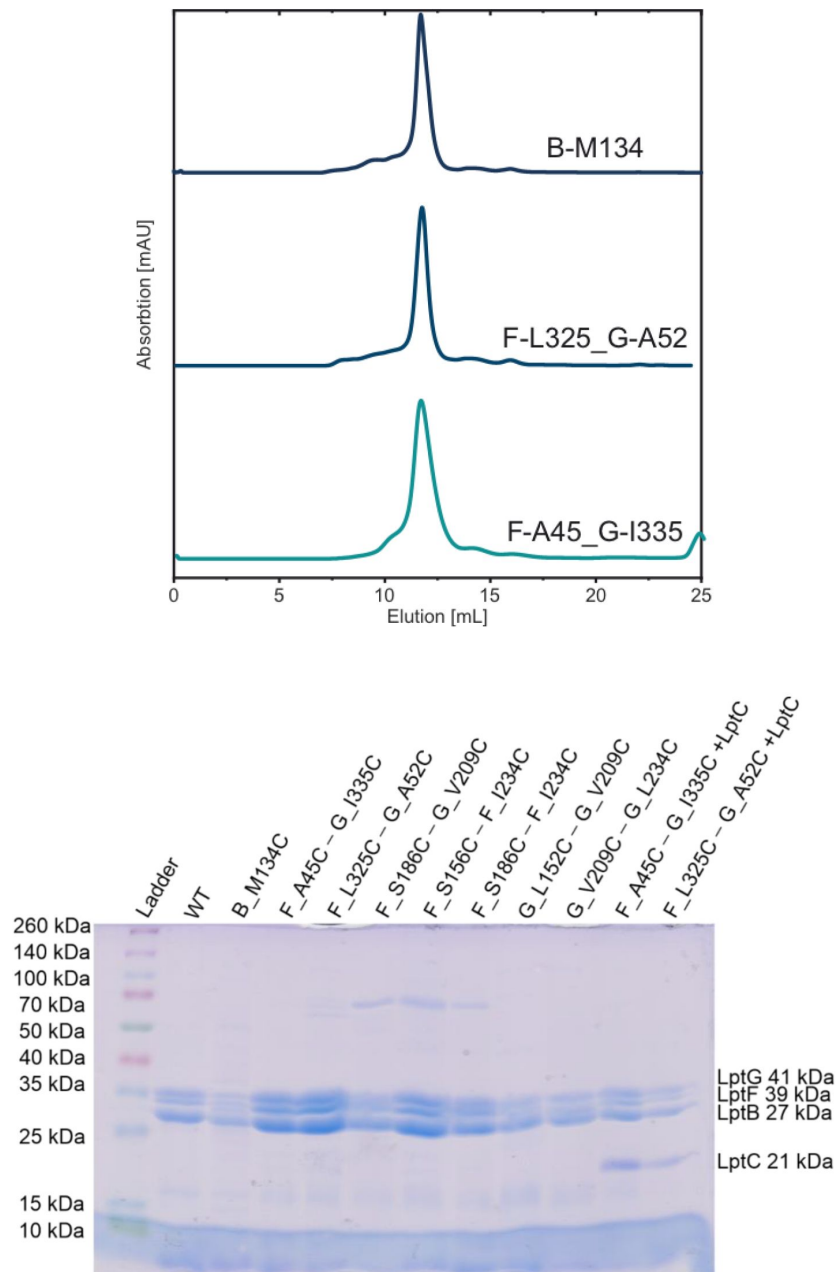


Figure S3.

Size-exclusion chromatography (SEC) and SDS-PAGE for WT and spin labeled cysteine variants of LptB₂FG and LptB₂FGC.

(Top panel) Typical SEC profiles for selected spin labeled variants as indicated are shown. (Bottom panel) SDS-PAGE gel. Proteins were collected from SEC column and the molecular weight of the subunits are indicated (LptG 41 kDa, LptF 39 kDa, LptB 27 kDa and LptC 21 kDa).

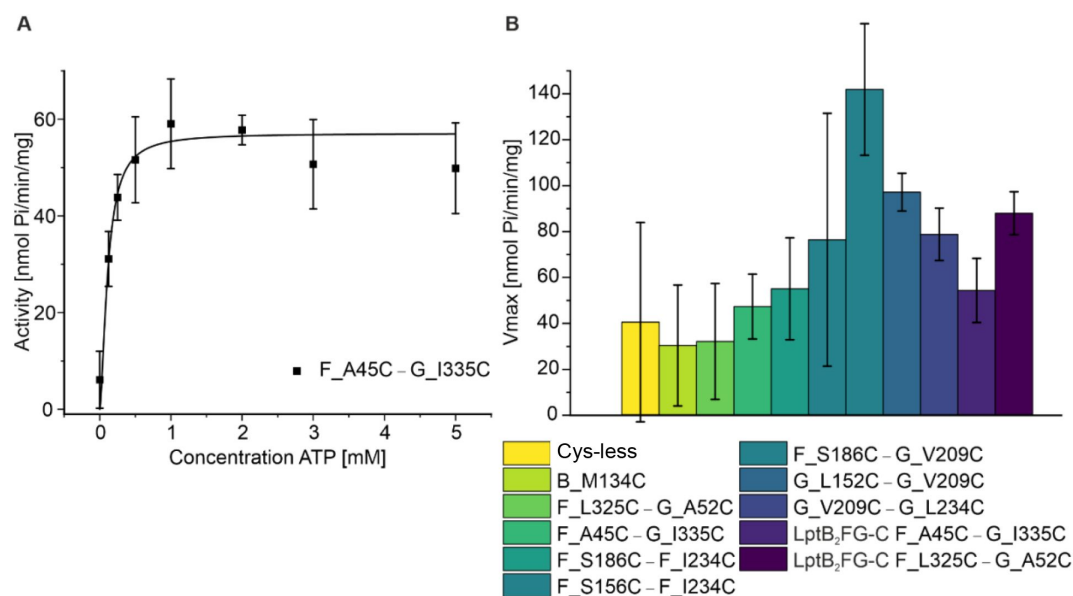


Figure S4.

ATPase assay for spin labeled variants of LptB₂FG and LptB₂FGC.

The functionality of the spin labelled proteins were characterized using ATPase assay and typical reaction curve is shown (left). The corresponding *V*_{max} values are shown (right). All the variants actively hydrolysed ATP, in line with the conformational changes observed from PELDOR/DEER experiments following vanadate-trapping (**Figures 2**–**6**). As the variants are spin labelled at different positions, a quantitative comparison between the values is omitted.

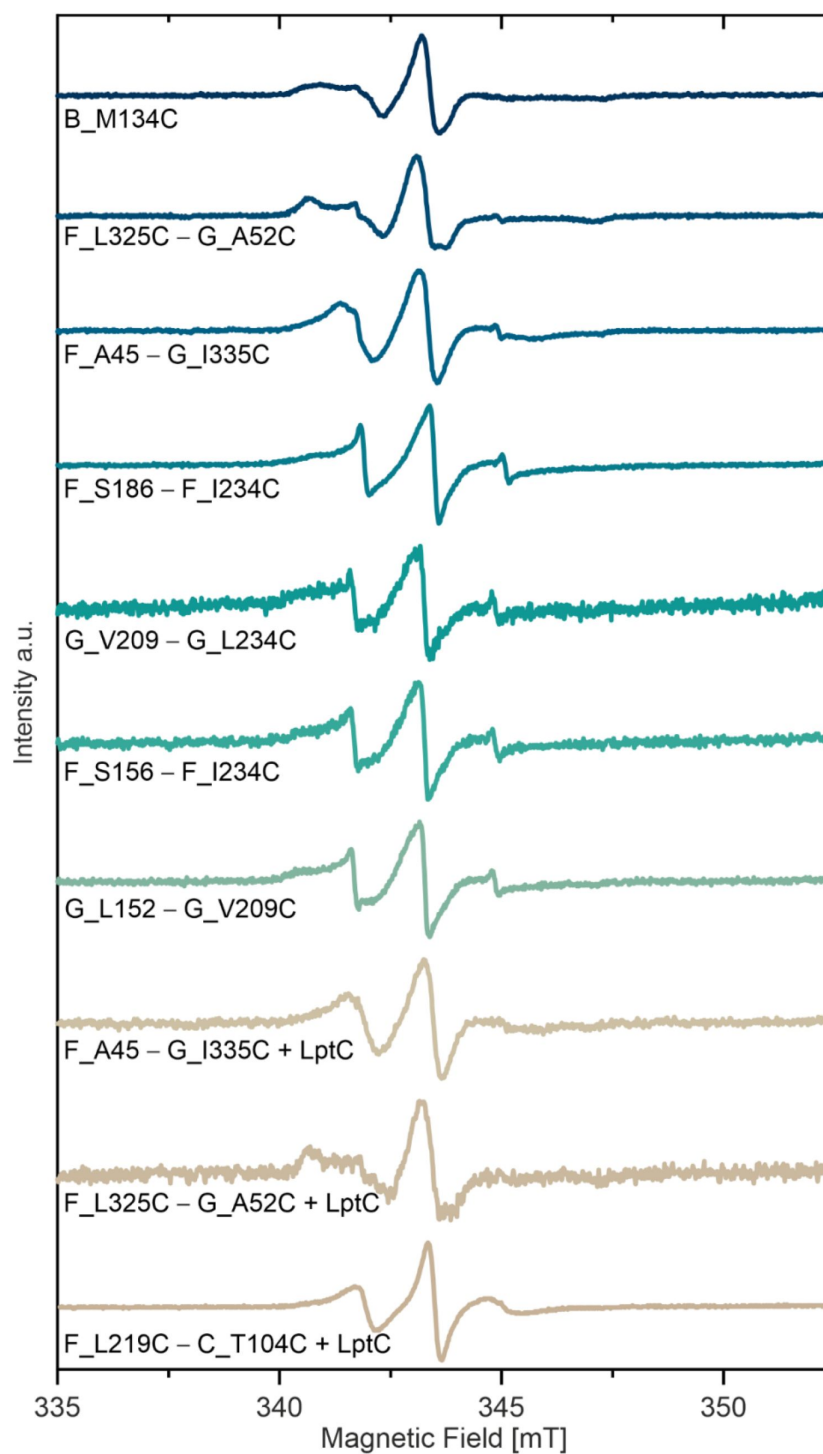


Figure S5.

Room temperature continuous wave ESR spectroscopy of MTSL labeled variants in micelles.

Spectra for spin labelled LptB₂FG and LptB₂FG-C variants in DDM micelles are shown.

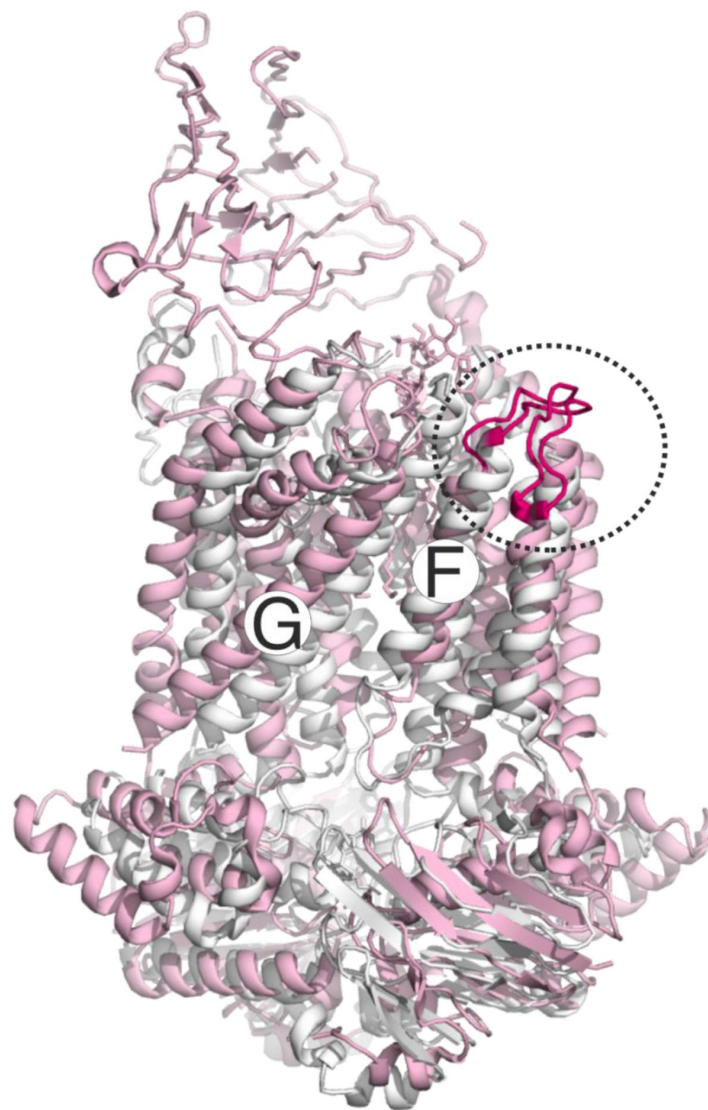


Figure S6.

Conformation of the loop carrying the spin labeled position 325 in LptF at the second lateral gate.

LptB₂FG apo (pink, PDB ID: 6MHU) and vanadate-trapped (grey, PDB ID: 6MHZ) structures are overlaid. The loop carrying the spin labelled position L325 is highlighted (magenta), which has a rather similar conformation in the two structures.

Figure S7.

Comparison of room temperature continuous wave ESR spectroscopy of MTSL labeled variants between DDM micelles and proteoliposomes (PLS).

Corresponding spectra for spin labelled LptB₂FG variants in liposomes and DDM micelles are shown.

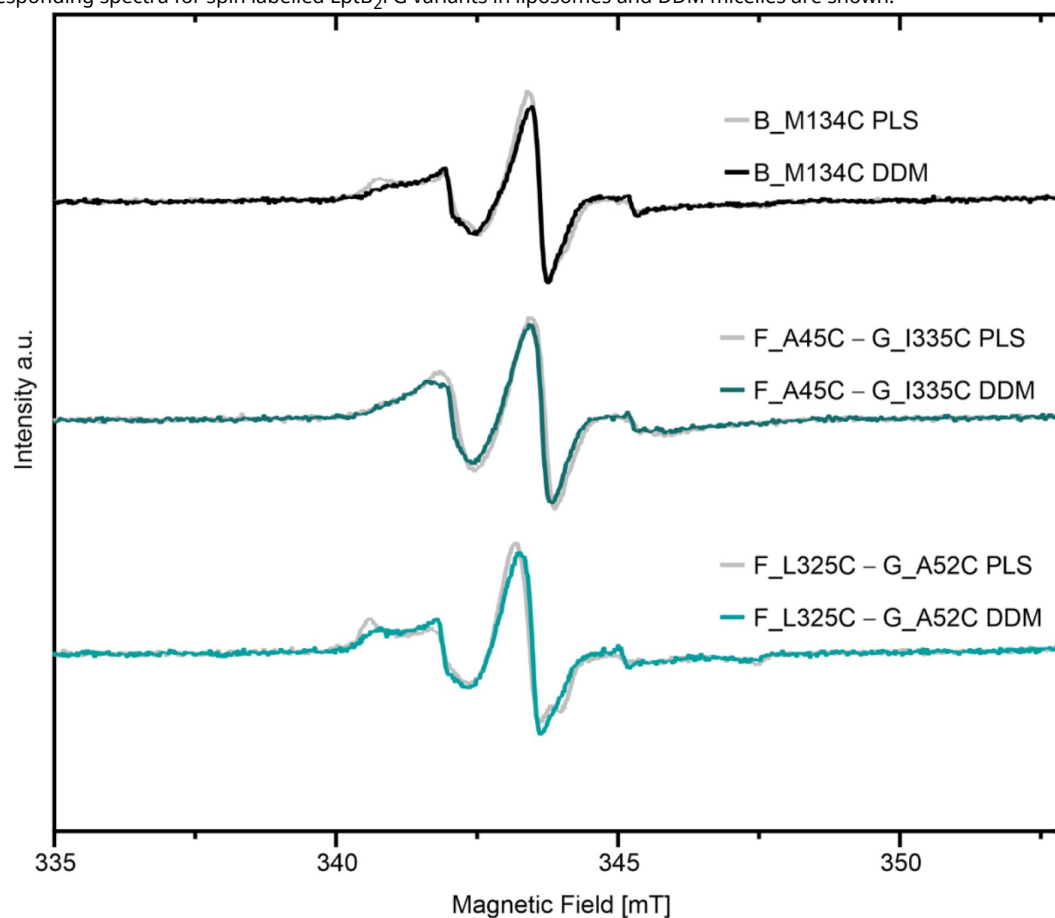


Table S1.

Spin labelling efficiency for the investigated cysteine variants.

A 10% error is estimated for the protein and spin concentrations.

Variant	Protein [μ M] ($\pm 10\%$)	Spin [μ M] ($\pm 10\%$)	Labelling efficiency [%] ($\pm 15\%$)
B_M134C PLS	60	125	96
F_A45C - G_I335C	37	72	98
F_L325C - G_A52C	37	69	93
F_S186C - G_V209C	31	70	112
F_S156C - F_I234C	70	130	93
F_S186C - F_I234C	31	61	99
G_L152C - G_V209C	46	87	95
G_V209C - G_L234C	34	64	94
F_A45C - G_I335C + LptC	29	58	100
F_L325C - G_A52C + LptC	31	62	100

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Reviewer #1 (Public Review):

Summary:

The current manuscript uses electron spin resonance spectroscopy to understand how the dynamic behavior and conformational heterogeneity of the LPS transport system change during substrate transport and in response to the membrane, bound nucleotide (or transition state analog) and accessory subunits. The study builds on prior structural studies to expand our molecular understanding of this highly significant bacterial transport system.

Strengths

This series of well-designed and well-executed experiments provide new mechanistic insights into the dynamic behavior of the LPS transport system. Notable new insights provided by this study include its indication of the spatial organization of the LptC domain, which was poorly resolved in structures, and how the LptC domain modulates the dynamic behavior of the gate through which lipids access the binding site. In addition, a mass spectrometry approach designed to examine LPS binding at different stages in the nucleotide-dependent conformational cycle provides insight into the order of operations of LPS binding and transport.

<https://doi.org/10.7554/eLife.99338.2.sa3>

Reviewer #2 (Public Review):

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and plays a critical role in bacterial virulence. The LPS export mechanism is a potential target for new antibiotics. Inhibiting this process can render bacteria more susceptible to the host immune system or other antibacterial agents. Given the rise of antibiotic-resistant bacteria, novel targets are urgently needed. The seven LPS transport (Lpt) proteins, A-G, move LPS from the inner to the outer membrane. This study investigated the conformational changes in the LptB2FG-LptC complex using site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy, revealing how ATP binding and hydrolysis affect the LptF β -jellyroll domain and lateral gates. The findings highlight the role of LptC in regulating LPS entry, ensuring efficient and unidirectional transport across the periplasm.

The β -jellyrolls are not fully resolved in the vanadate-trapped structure of LptB2FG and LptB2FGC. Therefore, the current study provides valuable information on the functional dynamics of these periplasmic domains, their interactions, and their roles in the

unidirectional transport of LPS. Additionally, the dynamic perspective of the lateral gates in LptFG in the presence and absence of LptC is another strength of this study. Moreover, at least in detergent samples, more comprehensive intermediates of the ATP turnover cycle are studied than in the available structures, providing crucial missing mechanistic details.

Other major strengths of the study include high-quality DEER/PELDOR distance measurements in both detergent and proteoliposomes, the latter providing valuable dynamics information in the lipid environment. The proteoliposome study is crucial since the previous structural study (Li, Orlando & Liao 2019) was done in rather small-diameter nanodiscs, which might affect the overall dynamics of the complex. It would have been beneficial if the investigators had reconstituted the complex in lipid nanodiscs with the same composition as proteoliposomes. The mixed lipid/detergent micelles provide an alternative. It seems the ATPase activity of the protein complex is much lower in detergent compared with lipid nanodiscs (Li, Orlando & Liao 2019). It is unclear how ATPase activity in proteoliposomes compares to that in detergent micelles.

Additionally, from previous structural studies and the mass spectrometry data presented here, LPS co-purifies and is already bound to the complex, thus the Apo state may represent the LPS-bound state without nucleotides.

<https://doi.org/10.7554/eLife.99338.2.sa2>

Reviewer #3 (Public Review):

Summary:

The manuscript by Dajka and co-workers reports the application of a biophysical approach to analyse the dynamics of the LptB2FG-C ABC transporter, involved in LPS transport across the cell envelope in *Escherichia coli*. LptB2FG-C belongs to a new class of ABC transporters (type VI) and is essential and conserved in several Gram-negative pathogens. Since LPS is the major component of the outer membrane of the Gram-negative cell and is responsible for the low permeability of this membrane to several antibiotics, a deep understanding of the mechanism and function of the LptB2FG-C transporter is crucial for the development of new drugs targeting Gram-negative pathogens.

Several structural studies have been published so far on the LptB2FG-C transporter, disclosing important aspects of the transport mechanism; nevertheless, lack of resolution of some regions of the individual proteins as well as the dynamic nature of the transport mechanism per se (e.g. the insertion and removal of the TM helix of LptC from the TMDs of the transporter during the LPS transport cycle) has greatly limited the understanding of the mechanism that couples ATP binding and hydrolysis with LPS transport. This knowledge gap could be filled by applying an approach that allows the analysis of dynamic processes. The DEER/PELDOR technique applied in this work fits well with this requirement.

Strengths:

In this study the authors provide some new pieces of information on the LptB2FG-C function and the role of LptC in the transporter using a technique that allowed them to appreciate missing intermediate conformations adopted by the proteins during the transport cycle.

The work is timely and well-conceived. The conclusions of the manuscript are supported by solid data and allow the authors to postulate a dynamic model for the mechanism of translocation of LPS across the inner membrane by the LptB2FGC complex.

<https://doi.org/10.7554/eLife.99338.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:**Reviewer #1:***Summary:*

The current manuscript uses electron spin resonance spectroscopy to understand how the dynamic behavior and conformational heterogeneity of the LPS transport system change during substrate transport and in response to the membrane, bound nucleotide (or transition state analog), and accessory subunits. The study builds on prior structural studies to expand our molecular understanding of this highly significant bacterial transport system.

Strengths

This series of well-designed and well-executed experiments provides new mechanistic insights into the dynamic behavior of the LPS transport system. Notable new insights provided by this study include its indication of the spatial organization of the LptC domain, which was poorly resolved in structures, and how the LptC domain modulates the dynamic behavior of the gate through which lipids access the binding site. In addition, a mass spectrometry approach designed to examine LPS binding at different stages in the nucleotide-dependent conformational cycle provides insight into the order of operations of LPS binding and transport.

We thank the reviewer for the very positive comments and highlighting the important findings from our study.

Reviewer #2 (Public Review):

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and plays a critical role in bacterial virulence. The LPS export mechanism is a potential target for new antibiotics. Inhibiting this process can render bacteria more susceptible to the host immune system or other antibacterial agents. Given the rise of antibiotic-resistant bacteria, novel targets are urgently needed. The seven LPS transport (Lpt) proteins, A-G, move LPS from the inner to the outer membrane. This study investigated the conformational changes in the LptB2FG-LptC complex using site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy, revealing how ATP binding and hydrolysis affect the LptF β jellyroll domain and lateral gates. The findings highlight the role of LptC in regulating LPS entry, ensuring efficient and unidirectional transport across the periplasm.

The β -jellyrolls are not fully resolved in the vanadate-trapped structure of LptB2FG and LptB2FGC. Therefore, the current study provides valuable information on the functional dynamics of these periplasmic domains, their interactions, and their roles in the unidirectional transport of LPS. Additionally, the dynamic perspective of the lateral gates in LptFG in the presence and absence of LptC is another strength of this study. Moreover, at least in detergent samples, more comprehensive intermediates of the ATP turnover cycle are studied than in the available structures, providing crucial missing mechanistic details.

We thank the reviewer for highlighting our major findings!

Other major strengths of the study include high-quality DEER distance measurements in both detergent and proteoliposomes, the latter providing valuable dynamics information in the lipid environment. However, lipid composition is not mentioned. The proteoliposome study is crucial since the previous structural study (Li, Orlando & Liao 2019) was done in rather small-diameter nanodiscs, which might affect the overall dynamics of the complex. It would have been beneficial if the investigators had reconstituted the complex in lipid nanodiscs with the same composition as proteoliposomes. The mixed lipid/detergent micelles provide an alternative. It seems the ATPase activity of the protein complex is much lower in detergent compared with lipid nanodiscs (Li, Orlando & Liao 2019). In the current study, ATPase activity in proteoliposomes is not provided. Also, the reviewer assumes cysteine-less (CL) constructs of the complex components were utilized. The ATPase assay on CL complex is not presented. Additionally, from previous structural studies and the mass spectrometry data presented here, LPS co-purifies and is already bound to the complex, thus the Apo state may represent the LPS-bound state without nucleotides.

The liposomes are made from E. coli polar lipid extract, which we added to the Materials and Methods part now. We could not yet perform the investigations in nanodiscs, which is one of our aims for future. The ATPase activity is lower in micelles and the reviewer is correct in that we did not perform/compare ATPase activity in proteoliposomes. The data denoted as wild-type (WT, Figure S4) corresponds to the cysteine-less (CL) variant, which is now corrected in the supporting information. As the reviewer commented, the mass spectrometry data reveal bound LPS in the apo-state. However, as seen from our results, ADP-Mg²⁺ state is similar to the apo state, thus in the cellular environment LPS may bind to this state as well.

The selection of sites to probe lateral gate 2, which forms the main LPS entry site, may pose an issue. Although the authors provide justification based on the available structures, one site (position 325 in LptF) is located on a flexible loop, and position 52 in LptG is on the neighboring transmembrane helix, separated by a potentially flexible loop from the gating TM1. These labeling sites could exhibit significant local dynamics, resulting in a broader distribution of distances and potentially masking the gating-related conformational changes.

Position 52 in LptG is located at the beginning of the neighboring transmembrane helix. As we have discussed in the manuscript, position 325 in LptF is located on a short loop connected to TM5. In the structures, this loop shows a very similar orientation (Figure S6). Further, the observed heterogeneity for the lateral gate-2 is considerably modulated into distinct conformation(s) upon LptC binding (Figure 6D-E). This would not be the case if this loop possesses any independent flexibility. Confirming these observations, the room temperature continuous wave ESR spectra revealed the least flexibility for this spin pair (Figure S5, S7). In view of the reasons and observations detailed above, we conclude that the local flexibility at the labelled sites might not make any significant contribution for the broad distribution observed at this gate in LptB2FG (Figure 4).

Reviewer #3 (Public Review):

Summary:

The manuscript by Dajka and co-workers reports the application of a biophysical approach to analyse the dynamics of the LptB2FG-C ABC transporter, involved in LPS transport across the cell envelope in Escherichia coli. LptB2FG-C belongs to a new class of ABC transporters (type VI) and is essential and conserved in several Gram-negative pathogens. Since LPS is the major component of the outer membrane of the Gram-negative cell and is responsible for the low permeability of this membrane to several

antibiotics, a deep understanding of the mechanism and function of the LptB2FG-C transporter is crucial for the development of new drugs targeting Gram-negative pathogens.

Several structural studies have been published so far on the LptB2FG-C transporter, disclosing important aspects of the transport mechanism; nevertheless, lack of resolution of some regions of the individual proteins as well as the dynamic nature of the transport mechanism per se (e.g. the insertion and removal of the TM helix of LptC from the TMDs of the transporter during the LPS transport cycle) has greatly limited the understanding of the mechanism that couples ATP binding and hydrolysis with LPS transport. This knowledge gap could be filled by applying an approach that allows the analysis of dynamic processes. The DEER/PELDOR technique applied in this work fits well with this requirement.

Strengths:

In this study, the authors provide some new pieces of information on the LptB2FG-C function and the role of LptC in the transporter. Notably, they show that:

- There is high heterogeneity in the conformational states of the entry gate of LPS in the transporter (gate-2) that are reduced by the insertion of LptC, and the heterogeneity observed is not altered by ATP binding or hydrolysis (as expected since LPS entry is ATP-independent).
- ATP binding induces an allosteric opening of LptF β -jellyroll domain that allows for LPS passage to the β -jellyroll of LptC, which is stably associated with the β -jellyroll of LptF throughout the cycle.
- The β -jellyroll of LptG is highly flexible, indicating an involvement in the LPS transport cycle.

The manuscript is timely and overall clear.

We thank the reviewer for the positive comments and highlighting our findings and the strength of DEER/PELDOR spectroscopy for characterizing the dynamics aspect of the LPS transport system.

Weaknesses:

I list my concerns below and provide suggestions that, in my opinion, should be addressed to reinforce the findings of this study.

(1) Protein complex controls: the authors assess the ATPase activity of the spin-labelled variants of their protein complexes to rule out the possibility that engineering the proteins to enable spin labelling could affect their functionality (Figure S4). It has been reported that the association of LptC to LptB2FG complex inhibits its ATPase activity. However, in the ATPase assay data shown in Figure S4, the inhibitory effect of the LptC TM is not visible (please compare LptB2FG F-A45C G-I335C and F-L325C G-A52C with and without LptC). This can lead to suspect that the regulatory function of LptC is missing in the LptC-containing complexes used in this work. I suggest the authors include wt LptB2FGC in the assay to compare the ATPase activity of this complex with wt LptB2FG. The published inhibitory effect of TM LptC has been observed in proteoliposomes. Since it is not clear from the paper if the ATPase assay in Figure 4 has been conducted in DDM or proteoliposomes, the lack of inhibitory effect could be due to the assay conditions. A comparative test could answer this question.

We could not observe the inhibitory effect of LptC on the ATPase activity of LptB2FG. As the reviewer pointed out, the primary reason is that we performed the assays in detergent micelles and not in proteoliposomes. For this reason, a comparison of the activity between (cysteine-less) LptB2FG and LptB2FG-C as the reviewer suggested would not be informative. As this information is not directly relevant for our current interpretations, we plan to perform those experiments in liposomes in the near future.

(2) Figure 2: NBD closure upon ATP binding to LptB2FG is convincingly demonstrated both in DDM micelles and proteoliposomes, validating the experimental system. However, since under physiological conditions, ATP binding should take place before the displacement of the TM of LptC (Wilson and Ruiz, Mol microbiol 2022), I suggest the authors carry out the experiments with LptC-containing complexes to investigate conformational changes (if any) that are triggered when ATP binding occurs before the TM displacement.

We thank the reviewer for the suggestion. These experiments are in our to do list and would be performed in the near future.

(3) Proteoliposomes: in the experiments shown in Figures 3 and 4, unlike those in Figure 2, measurements in proteoliposomes give different results from the experiments in DDM, showing higher heterogeneity. Could this be related to the presence (or absence) of LPS in liposomes? It is not mentioned in the materials and methods section whether LPS is present. Could the authors please discuss this?

We thank the reviewer for bringing out this interesting point. The liposomes are made from *E. coli* polar lipid extract. In the polar lipid extract, phosphatidylethanolamine (PE) is the predominant lipid component with minor amounts of phosphatidylglycerol (PG) and cardiolipin. Thus, the differences in the heterogeneity we observed in proteoliposomes might not be due to the presence of LPS. We added a short description on this aspect in the 'Discussion' part.

(4) The authors show large conformational heterogeneity in gate-2 (using the spin-labelled pair F-L325R1-G-A52R1) and suggest that deviation from the corresponding simulations could be due to the need for enhanced dynamics to allow for gate interaction with LPS or LptC. The effect of LptC is probed in the experiments shown in Figure 6, but I suggest the authors add LPS to the complexes to evaluate the possible stabilizing effect of LPS on the conformations shown in Figure 4.

This indeed is an important experiment, which we plan to do in the near future.

(5) Figure 6: the measurement of lateral gate 1 and 2 dynamics in the LptC-containing complexes clearly supports the hypothesis, proposed based on the available structures, that TM LptC dissociates from LptB2FG upon ATP binding. However, direct evidence of this movement is still missing. Would it be possible to monitor the dynamics of the TM LptC by directly labelling this protein domain? This would give a conclusive demonstration of the displacement during the ATPase cycle.

Yes, it should be possible to label LptC and monitor its position with respect to LptF or LptG. These experiments are in progress in our laboratory.

(6) LPS release assay: Figure 6 panels H-I-J show the MS spectra relative to LPS-bound and free proteins obtained from wt LptB2FG upon ATP binding and ATP hydrolysis conditions. From these spectra the authors conclude that LPS is completely released only

upon ATP hydrolysis. However, the current model predicts that LPS release into the Lpt bridge made by LptC-A-D is triggered by ATP binding. For this reason, I suggest the authors assess LPS release also from the LptB2FGC complex where, in the absence of LptA, LPS would be expected to be mostly retained by the complex under the same conditions.

These indeed are exciting experiments. LPS binding and release by LptB2FGC is in progress in our laboratories.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Page 2 typo: apo-sate should be apo-state

Thank you! We corrected the typo.

Can the authors clarify whether LPS is co-purified with the protein? Does it remain bound throughout the liposome reconstitution process?

Our mass spectrometry data show that LPS is co-purified with LptB2FG in micelles. However, we cannot yet verify the presence of bound LPS after reconstitution into proteoliposomes. We added a sentence in the last paragraph before Discussion as ‘Thus, LPS is co-purified with LptB2FG in micelles.’

Reviewer #2 (Recommendations for The Authors):

Several points require clarification:

(1) The reviewer would have benefited from access to the raw DEER traces. For instance, in Figure 4, the change in the raw data appears subtle. The differences between the Apo and vanadate-trapped states in b-DDM might be related to a lower signal-to-noise ratio in the Apo state.

We would be happy to share the raw DEER data upon request. The analysis is performed with the primary data, which also takes into account of the noise level for the calculating the confidence interval. Therefore, the distances with the 95% confidence interval are reliable to the extent as they are presented.

(2) The panel labels in Figures 2-4 do not match the legends.

Thank you! We corrected them.

(3) In Figure 2G, the authors state, "Overall, the ATP-induced closure as observed in micelles (and the structures) is maintained in the native-like lipid bilayers for the NBDs." This statement is technically incorrect since the vanadate-trapped state is not equivalent to the ATP+EDTA "ATP binding" state, which was not tested in proteoliposomes (PLS). The authors should have tested this condition for a few mutants in proteoliposomes. They should revise the manuscript to reflect this or provide evidence that the ATP+EDTA state is similar to the vanadate-trapped state in PLS.

We corrected the sentence as ‘Overall, the nucleotide-induced closure as observed in micelles (and the structures) is maintained in the native-like lipid bilayers for the NBDs.’

(4) The mutant F-L325R1_G-A52R1 is not optimal for probing gate 2. Specifically, position 325 in LptF is highly flexible, as indicated by the very broad distance distributions in Figure 4, and may hinder probing the associated conformational changes in this gate. Comparing the cryo-EM structures of this loop under different conditions (Figure S6) does not provide solid evidence for the lack of flexibility.

Position 52 in LptG is located at the beginning of the neighboring transmembrane helix. As we have discussed in the manuscript, position 325 in LptF is located on a short loop connected to TM5. In the structures, this loop shows a very similar orientation (Figure S6). Further, the observed heterogeneity for the lateral gate-2 is considerably modulated into distinct conformation(s) upon LptC binding (Figure 6D-E). This would not be the case if this loop possesses any independent flexibility. Confirming these observations, the room temperature continuous wave ESR spectra revealed the least flexibility for this spin pair (Figure S5, S7). In view of the reasons and observations detailed above, we conclude that the local flexibility of the labelled sites might not make any significant contribution for the broad distribution observed at this gate in LptB2FG (Figure 4).

(5) Regarding Figure 4B, the authors state, "In the vanadate-trapped and ATP samples, the major population is centered at 2 nm (which corresponds to the simulation on the vanadate trapped structure)". While the shift to shorter distances aligns with the structures, the average distance from the simulation is around 3 nm and does not correspond closely to the DEER distances of 2 nm.

Thank you for noting this point. We corrected the sentence as ‘In the vanadate-trapped and ATP samples, the major population is centred at 2 nm (which is closer to the simulation on the vanadate-trapped structure).’

(6) Regarding Figure 4D, the authors state, "Unlike the lateral gate-1 (and the NBDs), ADP-Mg²⁺ also induced a similar shift in the distance distribution." The reviewer believes that even without interaction with LptC, an equilibrium exists between two states in gate-2, and ATP binding or vanadate-trapping shifts the equilibrium to a shorter-distance population. Additionally, if the signal-to-noise ratio of the Apo state were similar to that of the ADP-Mg²⁺ state, similar distance distributions would have been observed for the Apo state.

We thank the reviewer for bringing out this excellent point. We thoroughly modified the corresponding section as ‘ADP-Mg²⁺ also gave a broad distribution comparable to the apo-state. Thus, in the apo-state this gate appears to exist in an equilibrium between the two conformations observed from the corresponding structures. ATP binding or vanadate-trapping shifts the equilibrium towards the collapsed conformation.’

(7) Defining the conformational dynamics of the b-jellyroll domains is one of the major strengths of this study. The LptF and LptG b-jellyroll domains exhibit high flexibility in detergent micelles. Unfortunately, none of the experiments were repeated in proteoliposomes to determine if this flexibility persists in a lipid environment.

As it is conceivable, it is truly beyond the scope of the current study to repeat all the measurements in liposomes. Currently we are extending those investigations to liposomes and would be able to provide more insights in the near future.

(8) Regarding Figure 6G, the authors claim, "Distances corresponding to the apo state are present possibly due to an incomplete vanadate trapping for this sample." It is

unlikely that vanadate trapping would be incomplete for just one sample. A repeat experiment is recommended.

We will update on this point is due time.

(9) Regarding the structural dynamics of the lateral gates, detergent micelles, and liposomes are vastly different environments. It is challenging to reach a consensus model based on data mostly derived from detergent micelles and only a few from proteoliposomes.

The observations in PLS are qualitatively similar to the micellar sample for the investigated positions (please see the first paragraph in “Discussion”). Further, our observations are in agreement with previous structural and biochemical data and further extent the mechanism in a coherent manner.

Reviewer #3 (Recommendations For The Authors):

Minor comments

(1) Figure legends: There are several mismatches between panel nomenclature and the corresponding descriptions in the legends. Please check the correspondence between panel identification and descriptions throughout the manuscript (for example, F-G and H-J in Figure 2; and I and H in Figure 3).

Thank you! We corrected them.

- Figure 6 legend: asterisk is in panel D and not C.

Corrected

- Panels E and F are not mentioned. Moreover, the spectra for vanadate trapped conformation of LptF219-LptC104 have not been given a letter.

Corrected

- A description of the different colors in the "Distance r" axis should be added to figure 2, 3, and 4 legends.

Corrected

- Please indicate the meaning of the black arrows in figure legends.

Corrected

(2) To improve data comprehension by the readers, the authors should indicate the relative spinlabelled pairs on the top of Figure 2, 3, and 4, as done for Figures 5 and 6.

Done

(3) Reference 56 is cited incorrectly in the reference list and refers to a study employing reconstituted LptB2FG complexes rather than isolated β -jellyroll domains.

Corrected

| (4) *Figure 3: How do the authors explain the evidence that ATP binding influences gate 1 conformational flexibility only in DDM micelles with respect of PLS? Is this something related to the release of LPS from the complex in different environments?*

We do not know whether this difference is related to LPS release. Therefore, we generally interpreted as an effect of the membrane environment.

| (5) *The initial sentence of the discussion looks somewhat incomplete, please correct it.*

Done

| (6) *To improve the readability of the paper, it could be useful to better focus the topic of the headings of the result paragraphs concerning the analysis of the individual lateral gates (for example, by indicating the name of the gate in the headings).*

Done

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