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The outer membrane translocon communicates with the inner membrane ATPase to stop lipopolysaccharide transport

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Abstract

The survival of Gram-negative bacteria depends on assembly of the asymmetric outer membrane, which creates a barrier that prevents entry of toxic molecules including antibiotics. The outer leaflet of the outer membrane is composed of lipopolysaccharide, which is made at the inner membrane and pushed across a protein bridge that spans the inner and outer membranes. We have developed a fluorescent assay to follow LPS transport across a bridge linking proteoliposomes that mimic the inner and outer membranes. We show that LPS is delivered to the leaflet of the outer membrane proteoliposome that corresponds to the outer leaflet of the membrane in a cell. Transport stops long before substrates at the inner membrane are exhausted. Using mutants of the transport machinery, we find that the final amount of LPS delivered into the membrane depends on the affinity of the outer membrane translocon for LPS. Furthermore, ATP hydrolysis depends on delivery of LPS into the outer membrane. Therefore, the transport process regulated by the outer membrane translocon can cause ATP hydrolysis in the inner membrane proteoliposome to stop. Negative feedback from the outer membrane to the inner membrane provides a mechanism for long distance control over LPS transport.

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Gram-negative bacteria have an unusual outer membrane that serves as a barrier to limit entry of toxic molecules.¹ This membrane is an asymmetric bilayer in which the inner leaflet consists of phospholipids while the outer leaflet is composed of lipopolysaccharide (LPS), a

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Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website. Experimental details, supporting figures and supporting tables. (PDF)

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large glycolipid containing six acyl chains that comprise the hydrophobic portion of the leaflet.^{2,3} During bacterial growth and division, phospholipid and LPS insertion into the inner and outer leaflets, respectively, must be coordinated to maintain outer membrane integrity. Here we report a quantitative assay to monitor LPS transport from one membrane to another, and show that activity of the LPS transport machinery is responsive to LPS levels in both membranes. Moreover, both ATP hydrolysis and LPS transport stop completely even when ATP and LPS are abundant at the inner membrane.

Lipopolysaccharide transport requires seven essential lipopolysaccharide transport proteins (Lpt), which form a bridge that spans the inner and outer membranes.^{4,5} At the inner membrane (IM), an ATP-dependent transporter, LptB₂FG, forms a complex with an additional membrane protein LptC.^{6–10} At the outer membrane (OM), the lipoprotein LptE forms a plug-in-barrel complex with the beta-barrel protein LptD.^{11–15} The soluble periplasmic Lpt protein, LptA, bridges these two membrane complexes.¹⁶ LptB₂FG uses multiple rounds of ATP hydrolysis to power sequential transport steps, including LPS extraction from the IM, release to LptC, movement along the LptA protein bridge and, finally, LptDE-dependent insertion of LPS into the outer leaflet of the OM.¹⁷

To understand the factors that influence LPS transport, we developed a quantitative method to monitor transport rates. We previously reported a biochemical system that recapitulates membrane-to-membrane transport of LPS using proteoliposomes containing either purified LptB₂FGC and LPS (IM proteoliposomes), or LptDE (OM proteoliposomes) complexed with LptA.⁵ To demonstrate transport, we monitored crosslinking of LPS to photo-active sites in Lpt components. However, our method did not enable quantification of transport rates, nor did it allow observation of insertion of LPS into the OM proteoliposome. To enable quantitation, we have prepared dansylated polymyxin B nonapeptide (dansyl-PMBN, Figure S1), a natural product derivative that binds LPS.¹⁸ Because the fluorescence of the dansyl group increases in a hydrophobic environment (*e.g.*, a membrane),¹⁹ the dansyl-PMBN probe can be used to quantify increasing LPS abundance in a membrane (Figure 1A). We chose to work with *E. coli* Ra-LPS, the variant of LPS present in the laboratory strain, K-12 *E. coli* which has been structurally characterized, lacks O-antigen but contains all core sugars (Figure 2C).

We encapsulated dansyl-PMBN into proteoliposomes containing LptDE to be a reporter for LPS pathway flux (Figure 1A, Figure S3, Table S1, S2). Fluorescence signal increased when proteoliposomes containing LptDE with encapsulated dansyl-PMBN (OM-dansyl proteoliposomes) were incubated with IM proteoliposomes, LptA, and ATP (Figure 1B, Figure S4). This fluorescence increase provides the first evidence for LPS insertion into the OM proteoliposome, demonstrating that the reconstituted LptDE translocon is functional. When we monitored the fluorescence signal over time, we observed an initial lag phase (5 min), a region of linear increase (10–30 min), and signal saturation (60 min). No fluorescence increase was observed if ATP, LptA, or the IM protein complex was omitted from the biochemical system. (Figure 1B, Figure S5). Therefore, our fluorescent assay detects ATP-driven LPS transport from the IM to the OM proteoliposomes via insertion of LPS into the probe-accessible inner leaflet of the OM proteoliposomes. We also confirmed that full-length LPS containing O-antigen can be transported in our system (Figure S6).

The fluorescence-based assay allows comparison of the rates of LPS transport for different conditions using the slopes of the linear range, enabling identification of factors that influence the rate of transport. We prepared IM proteoliposomes containing variable amounts of LPS to generate a standard curve to correlate fluorescence with the amount of LPS transported (Figure S7). We tested the effect of LPS concentration on transport rate (Figure 2A, B). Surprisingly, as we increased the concentration of Ra-LPS in the IM proteoliposome above 62.5 μ M, the rate of transport decreased (Figure 2A). At lower LPS concentrations, the transport rate increased with increasing LPS (up to 62.5 μ M, Figure 2B). LPS has an extremely low (pM) critical micelle concentration (CMC) and a propensity to self-associate due to its ability to form very strong lateral interactions.^{20,21} One explanation for the inverse correlation between substrate concentration and transport rate at higher LPS concentrations is that lateral interactions increase with concentration, resulting in a higher barrier for extraction from the membrane. If so, truncating the structure of the LPS molecule to reduce its lateral interactions should increase the rate of transport.

We compared a set of truncated LPS variants to examine how structure affects the rate of transport. LPS contains a disaccharide backbone with six fatty acyl chains (lipid A) and an oligosaccharide core with up to 15 sugar residues that can be modified with additional sugars (O-antigen) (Figure 2C).^{2,22} We prepared IM proteoliposomes containing four variants of LPS (Ra, Rc, Re, lipid A), the shortest of which, lipid A, is a hexaacylated disaccharide. All four variants were transported, demonstrating that the lipid A structure without any core sugars is sufficient for transport (Figure 2C, Figure S8). Notably, the two smallest LPS variants, lipid A and Re-LPS, were transported faster than the variants with more core sugars. Lipid A and Re-LPS have higher CMCs than the larger LPS variants consistent with the hypothesis that efficient LPS extraction from the IM requires disruption of lateral interactions between LPS molecules.

We wondered how binding of LPS to the OM translocon would affect transport. We previously characterized two point mutations in LptE (K136D or R91D-K136D) that affect its ability to bind to and disaggregate LPS.²³ The LptE-K136D single mutant cannot bind LPS as effectively as wild-type LptE; the binding of LptE R91D-K136D double mutant to LPS is almost completely ablated. To test the effect of these mutations on transport we generated LptDE complexes containing these variants of LptE. Both LptE variants exhibited decreased rates of LPS transport (Figure 2C). This suggests that LptE binding to LPS is essential for efficient insertion into the OM.

A notable feature of the LPS transport reactions is that fluorescence stopped increasing after 30–60 min (Figure 1B, Figure 2A–C). A trivial explanation would be that one of the components of the system (dansyl-PMBN, LPS, or ATP) was limiting. However, the maximum fluorescence for probe binding to LPS in liposomes is well above the fluorescence observed in transport reactions (Figure S6), so the fluorescence was not limited by either the concentration of dansyl-PMBN or its ability to bind LPS. The initial LPS concentration was not limiting because fluorescence saturated at the same concentration of LPS transported whether the initial concentration was 62.5 μ M or 125 μ M LPS (Figure 2A). To test whether ATP concentration was limiting, we repeated the transport experiments at varying concentrations of ATP (Figure 3A, Figure S9, Table S3). Although ATP

concentration affected how quickly fluorescence saturated, the LPS concentration at the plateau was the same. The simplest explanation is that LPS transport stopped for a reason other than substrate depletion.

To confirm that LPS transport stopped, we used a photoaffinity crosslinking assay that captures LPS in an intermediate transport state.²⁴ We observed crosslinking between LPS and LptD containing a photoactive unnatural amino acid only at time points where, in our fluorescent assay, the signal was still increasing (Figure 3B). That no photocrosslinking was detected subsequently implies that no LPS was passing through the LPS binding site consistent with LPS transport having stopped.

To ask whether the activity of the ATPase in the IM proteoliposome is affected when LPS transport into the OM proteoliposome stops, we simultaneously monitored LPS transport and phosphate release (Figures 2C and 4A). ATP hydrolysis has a similar time dependence as LPS transport. The similarity between the time dependence of signal saturation indicates that ATP hydrolysis is coupled to LPS transport. Both LptE mutants exhibited a decrease in ATPase activity that mirrors decreased LPS transport (Figure 4A). We find it remarkable that ATP hydrolysis in one proteoliposome is affected by changing a protein in a separate proteoliposome.

Our findings imply that a signal turns off LPS transport. One explanation is that additional LPS cannot be inserted into the OM past a threshold concentration of LPS. Alternatively, since the reconstituted system does not allow for simultaneous transport of phospholipids, LPS transport could be limited by an imbalance between lipids in the inner and outer leaflets of the proteoliposomes, generated as LptDE translocates LPS. To distinguish between these possibilities, we prepared OM-dansyl proteoliposomes preloaded with varying concentrations of LPS and containing either wild-type or mutant LptE, and monitored transport (Figure 4B). As observed in Figure 2D, the mutant LptE variants stop transporting LPS at a lower final concentration in the OM. However, none of the LptDE variants could transport LPS into OM-dansyl proteoliposomes preloaded with LPS. If insertion depended primarily on coordinating simultaneous growth of the membrane leaflets, we would have seen transport of LPS into the proteoliposomes preloaded with LPS. We conclude that additional LPS cannot be inserted into the OM past a threshold concentration of LPS. This concentration depends on the structure of the translocon and its affinity for substrate.

We have fully reconstituted the LPS transport machine and demonstrated translocation of LPS across a membrane. Using a fluorescent probe that detects translocon-dependent delivery of LPS into the membrane, we can assess different substrates as well as defective translocon components. The amount of LPS delivered depends on the affinity of the translocon for its substrate. We propose a model wherein the translocon exists in at least two states, one of which turns off delivery to the OM. The inability to deliver LPS into the OM somehow results in a cessation of ATP hydrolysis. Our results explain observations in cells that translocon mutants do not properly assemble LPS in the OM. How the lipopolysaccharide transporter achieves coupling between the ATPase that powers transport, and delivery of LPS in a separate cellular compartment is not yet known. Long-range control

of LPS delivery is crucial to prevent the cell from wasting resources when the outer membrane is filled.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A fluorescent assay monitors LPS transport. (A) Schematic depicting assay design. The dansyl-PMBN probe selectively binds LPS and has a long LPS concentration-dependent linear range (Figure S2). The inner leaflet of the proteoliposome containing LptD/E corresponds to the outer leaflet of the OM *in vivo*. (B) Ra-LPS transport requires all seven Lpt proteins and ATP.

Figure 2.

Transport rates are affected by the concentration and size of the LPS cargo and transport requires that LptE bind LPS. (A)-(C) IM proteoliposomes were incubated with OM-dansyl proteoliposomes, LptA, and ATP, and assayed over time for each condition. (A-B) IM proteoliposomes containing variable amounts of Ra-LPS. (C) IM proteoliposomes containing structural variants of LPS (Ra, Rc, Re, lipid A). (D) OM proteoliposomes containing variants of LptE with reduced binding affinity for LPS.

Figure 3.

LPS transport has stopped when fluorescence saturates. (A) IM proteoliposomes containing 125 μM Ra-LPS were incubated with OM-dansyl proteoliposomes, LptA, and varying concentrations of ATP. Dotted box indicates the linear range of fluorescence. (B) The linear range of fluorescent signal. Transport rate varies with ATP concentrations. (C) IM proteoliposomes containing Ra-LPS were incubated with OM proteoliposomes containing LptD labeled with a photoactive amino acid, LptA, and two concentrations of ATP. Signal was detected by immunoblotting using an αLPS antibody. αLptD was used as a loading control.

Figure 4.

Buildup of LPS in the OM proteoliposome affects translocon function and stops ATP hydrolysis. (A) ATP hydrolysis was monitored simultaneously with transport activity for IM proteoliposome incubated with LptA and OM-dansyl proteoliposomes containing W T or mutant LptE variants. (B) OM-dansyl proteoliposomes containing W T or mutant LptE variants and preloaded with varying concentrations of Ra-LPS were incubated with IM proteoliposomes, LptA, and ATP.