Cytoplasmic ATP Hydrolysis Powers Transport of Lipopolysaccharide Across the Periplasm in *E. coli*

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Millions of molecules of lipopolysaccharide (LPS) must be assembled on the *Escherichia coli* cell surface each time the cell divides. The biogenesis of LPS requires seven essential lipopolysaccharide transport (Lpt) proteins to move LPS from the inner membrane through the periplasm to the cell surface. However, no intermediate transport states have been observed. We developed methods to observe intermediate LPS molecules bound to Lpt proteins in the process of being transported in vivo. Movement of individual LPS molecules along these binding sites required multiple rounds of adenosine triphosphate (ATP) hydrolysis in vitro, which suggests that ATP is used to push a continuous stream of LPS through a transenvelope bridge in discrete steps against a concentration gradient.

The outer membrane of Gram-negative bacteria is an asymmetric bilayer, with the inner leaflet composed of phospholipids and the outer leaflet composed of lipopolysaccharide (LPS). The LPS layer allows Gram-negative bacteria to survive in harsh environments and in the presence of many antibiotics. However, phospholipids and LPS do not spontaneously assemble into an asymmetric bilayer, and the cell devotes substantial resources to synthesizing LPS at the inner membrane and assembling it in the outer membrane (*1*). The process by which hydrophobic LPS molecules are transported across two membranes and the intervening aqueous periplasmic space is not understood, but it is known to be mediated by seven essential Lpt (lipopolysaccharide transport) proteins. The heteromeric ATP-binding cassette (ABC) transporter LptBFG forms a complex in the inner membrane with a membrane-bound protein, LptC (Fig. 1A) (*2*–*5*). Using homologous domains, the C terminus of LptC interacts with the N terminus of LptA, and the C terminus of LptA interacts with the N-terminal periplasmic domain of LptD (*6*–*9*). The resulting head-to-tail oligomer produced creates a transenvelope protein bridge that connects the inner-membrane Lpt components with the outer-membrane LPS translocon (LptD and LptE) (*6*, *10*–*15*). Defects in any of the Lpt proteins cause LPS to accumulate in the outer leaflet of the inner membrane (*2*, *16*). However, the mechanism of LPS transport has been difficult to study because it involves two membranes and seemingly cannot be broken into different steps.

To elucidate the mechanism of LPS transport, we first sought to identify sites where LPS interacts with the Lpt proteins in vivo. We incorporated an unnatural amino acid containing a photo–cross-linker (*p*-benzoylphenylalanine, *p*BPA) at 23 positions in LptC and 14 positions in LptA (*17*–*20*). Four of the LptC mutants and five of the LptA mutants formed cross-links to LPS in an ultraviolet light (UV)–dependent manner (Fig. 1, B and C). LptC adducts contained the cross-linker at positions 47, 78, 172, and 182 (Fig. 1B), whereas LptA adducts contained the cross-linker at positions 32, 36, 95, 114, and 116 (Fig. 1C). Except for residue 47 in LptC, all of these LPS adducts formed on the inside of the β-jellyroll structures. Residue 47 in LptC is in a disordered region of the protein (*9*), which may become ordered when it interacts with the LptBFG complex or LPS. No cross-links from side chains on the outer surface of the β jellyroll of LptC or LptA were observed. Thus, we propose that LPS specifically binds inside these proteins and transits the periplasm on the inside of the β jellyroll of these proteins.

To determine whether LptC or LptA alone can extract LPS from the inner membrane or whether cross-linking to LPS is dependent on the entire transenvelope complex, we manipulated the expression levels of the Lpt components. We overexpressed LptC or LptA alone or in combination with the other inner-membrane Lpt proteins (i.e., LptBFG or LptBFGC), reasoning that LptC and LptA would mostly not reside in Lpt complexes if they were overexpressed individually. Cross-linking of LPS to LptC at position 47, but not at positions 78, 172, or 182, increased substantially when LptBFG was co-overexpressed (Fig. 2A). Thus, when LptA, LptD, and LptE are limiting, LPS accumulates near residue 47 of LptC. Position 47 may be located near a strong binding site for LPS, whereas the other cross-linking positions may reflect more transient interactions that occur during the movement of LPS through fully assembled bridges. Because LPS accumulation at the binding site around residue 47 depends on LptBFG, it is also clear that LptC cannot extract LPS directly from the inner membrane unaided.

All five LptA mutants showed increased cross-linking with LPS when they were co-overexpressed with LptBFGC (Fig. 2B). Thus, LptA cannot receive LPS directly from the inner membrane, but requires LptBFGC complex. LPS must migrate out of the inner membrane in an ordered process that requires LptBFG to get to LptC and LptBFGC to get to LptA. Because LPS transport between these proteins can occur in incomplete Lpt bridges, it was possible to develop a biochemical system to separate the individual step of LPS release from the membrane from that of the transport step along the Lpt bridge.

To study the ATP dependence of LPS extraction from the inner membrane, we first prepared spheroplasts containing overexpressed LptC(T47*p*BPA) and LptBFG by disrupting the outer membrane and cell wall with ethylenediaminetetraacetic acid (EDTA) and lysozyme. The inner membrane was then permeabilized by osmotic shock to introduce buffer with or without ATP into the cytoplasm to generate right-side-out (RSO) membrane vesicles (*21*–*25*). These vesicles were then irradiated with UV light. Substantial LPS cross-linking to residue 47 of LptC was only observed when LptBFG was co-overexpressed and ATP was present (Fig. 3A). A small amount of LPS was cross-linked to LptC in the absence of ATP, but this amount did not increase over time (Fig. 3A). Presumably this small amount of LPS was present before generating the RSO fraction. Because cross-linking increased as a function of time in the presence of ATP, transfer of LPS from the inner membrane to the membrane-bound LptC appears to require energy.

We next addressed whether LPS could be transferred to the periplasmic component, LptA, in this in vitro system. This reconstitution requires release of LPS from a membrane fraction to a soluble periplasmic protein (fig. S1). Purified LptA(I36*p*BPA), which cross-linked LPS in vivo, was added to RSO vesicles and analyzed for cross-linking to LPS. LPS cross-linked to LptA over time only in the presence of ATP and when all four inner-membrane components were overexpressed (Fig. 3B). Notably, LPS did not cross-link to LptA in the absence of LptC overexpression; this finding indicates that LPS must first be transferred to LptC before being transferred to LptA, which is consistent with previous work (*9*).

To confirm that the soluble periplasmic bridge component assembled in this in vitro system reflects the in vivo assembly process, we tested the specificity of this cross-linking. An LptA mutant, LptA(H37*p*BPA), interacts directly with LptC (*6*) but not with LPS in vivo (Fig. 1C). Residues Ile^{36} and His^{37} are both located on the edge of the β jellyroll that interacts with LptC,

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but their side chains are oriented in opposite directions (Fig. 3C). In this RSO vesicle reconstitution, the His^{37} mutant cross-linked to LptC (Fig. 3D). However, although His^{37} is directly adjacent to Ile^{36}, His^{37} did not cross-link to LPS in the presence of ATP (Fig. 3B). Thus, our in vitro reconstitution cleanly recapitulates the specific protein-protein and the protein-substrate interactions observed in vivo. Because the binding of LptA to LptC was rapid and did not depend on time or the presence of ATP (Fig. 3D), energy appears not to be required for assembly of the bridge, but is required to transport LPS from the inner membrane to LptA.

A small amount of LPS accumulated in LptC in the absence of ATP (Fig. 3A) but did not spontaneously move to LptA (Fig. 3B). We thus asked whether LPS transfer from LptC to LptA requires a separate energetic step. We incubated RSO vesicles overexpressing LptBFG-LptC(T47*p*BPA) with ATP to accumulate LPS in LptC, and then added purified LptA(I36*p*BPA) with or without an adenosine triphosphatase (ATPase) inhibitor, vanadate, before irradiating the samples. LPS did not cross-link to LptA in the inhibitor-treated samples, which shows that LPS transfer from LptC to LptA required energy (Fig. 4A). In the absence of vanadate, LptC–cross-linked adducts with LPS were not decreased even when LPS transferred to LptA, which suggests that the binding sites for LPS in the periplasmic bridge are always filled with LPS during its transport. Thus, at least two energy-dependent steps are involved in LPS transport: one during extraction from the membrane to the periplasmic domain of the membrane-bound LptC, and another during transfer of LPS

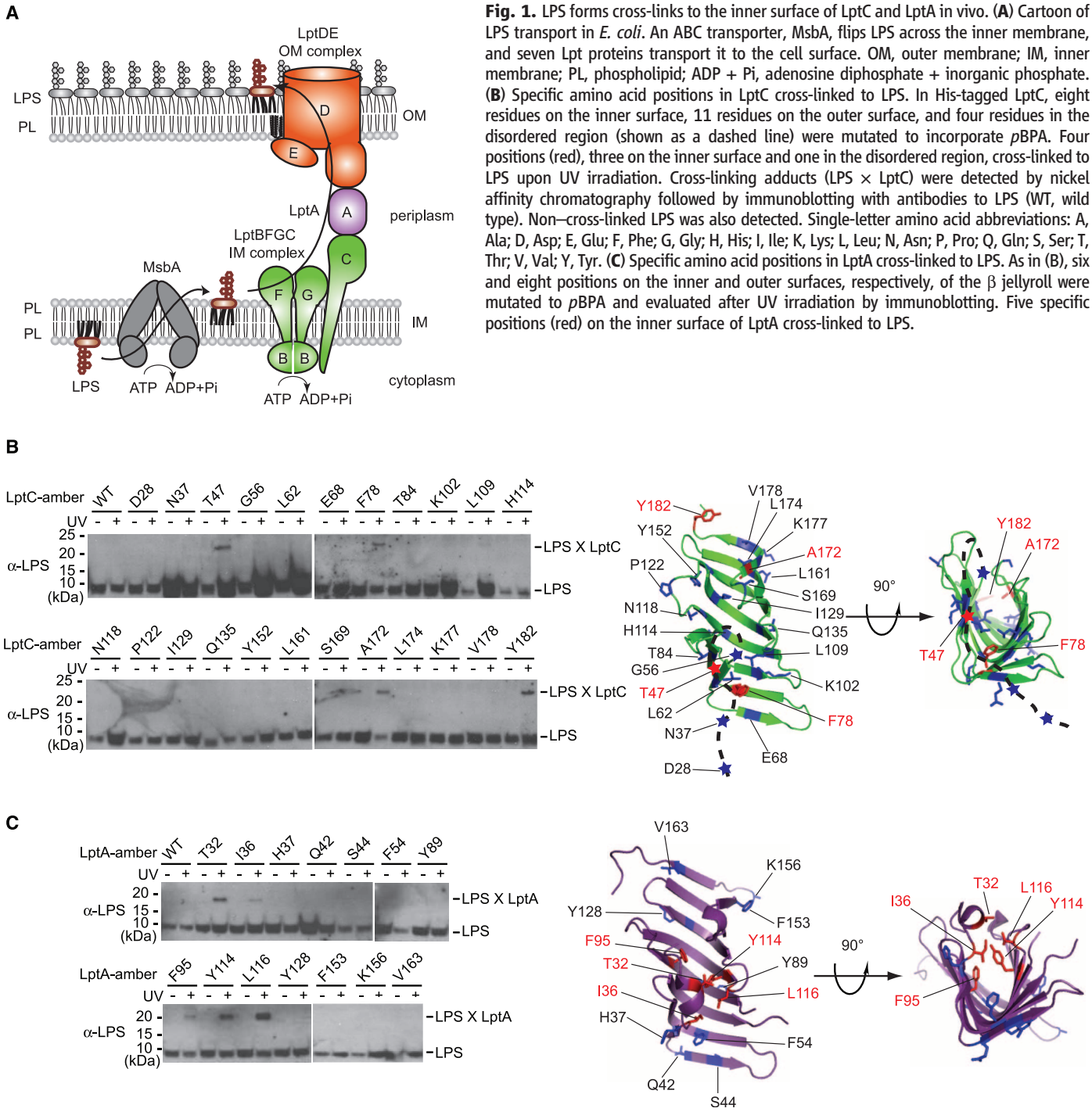

Fig. 1. LPS forms cross-links to the inner surface of LptC and LptA in vivo. (**A**) Cartoon of LPS transport in *E. coli*. An ABC transporter, MsbA, flips LPS across the inner membrane, and seven Lpt proteins transport it to the cell surface. OM, outer membrane; IM, inner membrane; PL, phospholipid; ADP + Pi, adenosine diphosphate + inorganic phosphate. (**B**) Specific amino acid positions in LptC cross-linked to LPS. In His-tagged LptC, eight residues on the inner surface, 11 residues on the outer surface, and four residues in the disordered region (shown as a dashed line) were mutated to incorporate *p*BPA. Four positions (red), three on the inner surface and one in the disordered region, cross-linked to LPS upon UV irradiation. Cross-linking adducts (LPS × LptC) were detected by nickel affinity chromatography followed by immunoblotting with antibodies to LPS (WT, wild type). Non–cross-linked LPS was also detected. Single-letter amino acid abbreviations: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; S, Ser; T, Thr; V, Val; Y, Tyr. (**C**) Specific amino acid positions in LptA cross-linked to LPS. As in (B), six and eight positions on the inner and outer surfaces, respectively, of the β jellyroll were mutated to *p*BPA and evaluated after UV irradiation by immunoblotting. Five specific positions (red) on the inner surface of LptA cross-linked to LPS.

from LptC to LptA. These steps may occur synchronously. We propose a model for LPS transport in which ATP hydrolysis in the cytoplasm provides energy to push LPS molecules in discrete steps across the bridge (Fig. 4B).

The mechanism of LPS transport is somewhat similar to that of efflux pumps, which also form transenvelope bridges and use energy to drive a small molecule through a continuous channel to the exterior environment (*26*–*28*). The model for efflux pumps suggests that only one energy-dependent step drives one toxic molecule out of the cell. This requirement reflects the fact the noxious molecules are released from the internal three-dimensional reservoir into the channel of the pump, which is continuous with the infinite exterior environment; diffusion can efficiently carry the molecule away. Movement of LPS from the inner membrane to the outer membrane across the periplasm proceeds through a series of energy-dependent steps as sequential molecules are pushed in a continuous stream through the bridge. The problem of LPS transport is different from drug efflux in that LPS must move from a two-dimensional reservoir at the inner membrane to another two-dimensional reservoir at the cell surface in opposition to the concentration gradient. Multiple rounds of ATP hydrolysis are required to increase the efficiency of unidirectional transport. If transport across the periplasmic bridge requires that multiple binding sites on Lpt proteins be filled with LPS during transport, it may be possible to design inhibitors that destroy the pumping mechanism by introducing air in the line.

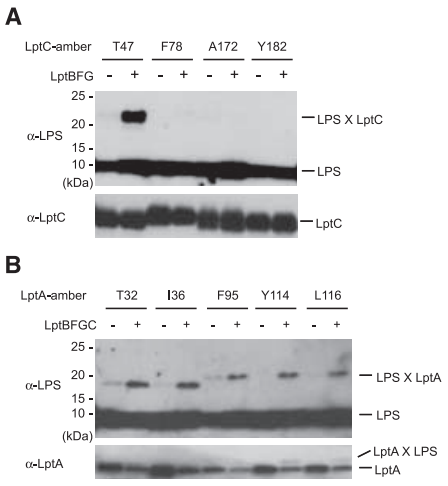

Fig. 2. LPS cross-linking in LptA and LptC depends on the inner-membrane components of the Lpt machine. (**A**) LPS accumulates near residue Thr[47] of LptC in LptBFGC complexes. LptC mutants that cross-link to LPS were overexpressed with or without co-overexpression of LptBFG. Cross-linking was detected similarly to Fig. 1B with antibodies to LPS and LptC. Cross-linking at position 47 substantially increases when LptBFG is co-overexpressed. (**B**) LPS accumulates at several positions of LptA in a LptBFGC-dependent manner. In a method analogous to that in (A), LptA mutants that cross-link to LPS were overexpressed with or without co-overexpression of LptBFGC. Cross-linking at all five positions increases when LptBFGC is co-overexpressed.

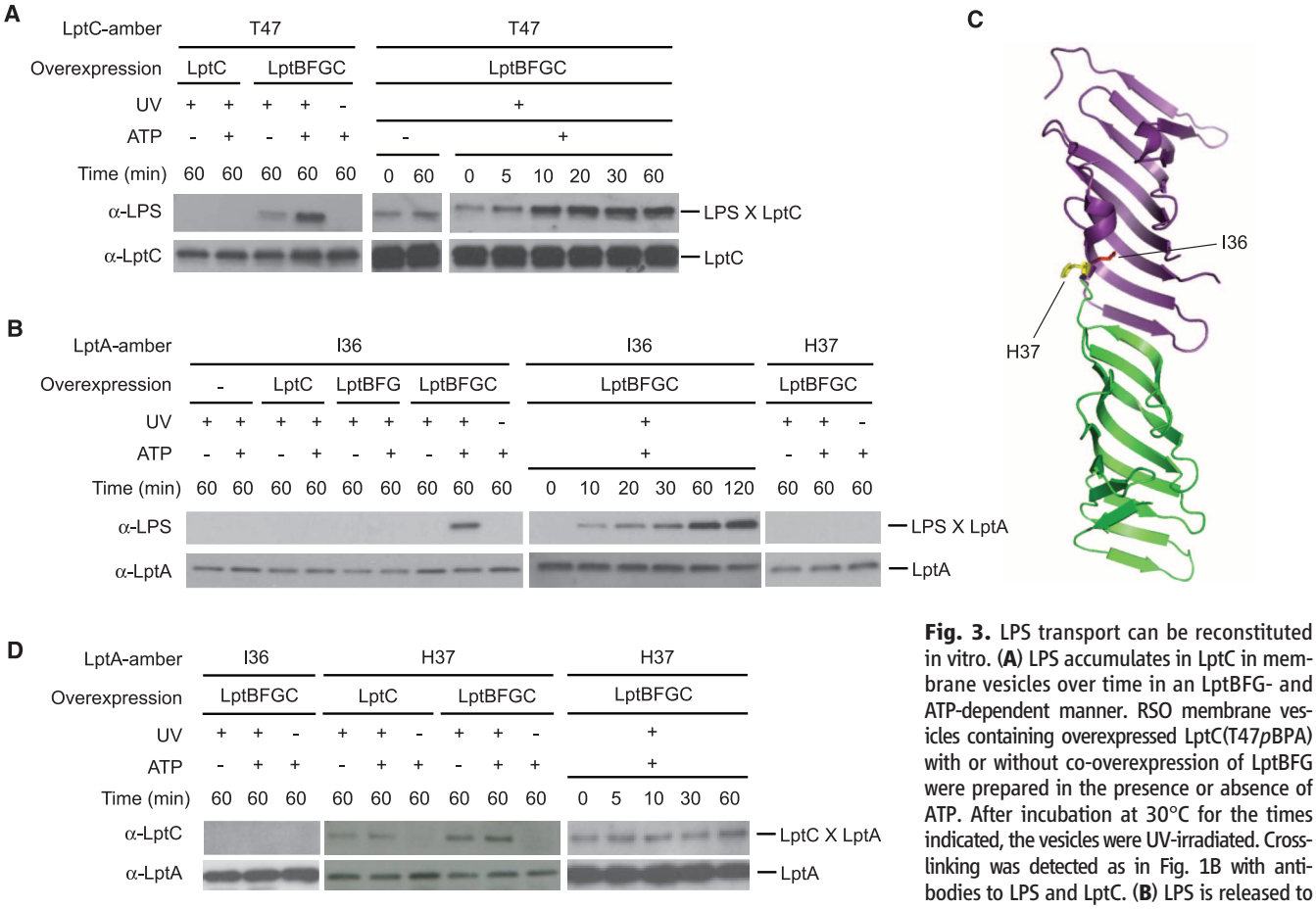

Fig. 3. LPS transport can be reconstituted in vitro. (**A**) LPS accumulates in LptC in membrane vesicles over time in an LptBFG- and ATP-dependent manner. RSO membrane vesicles containing overexpressed LptC(T47*p*BPA) with or without co-overexpression of LptBFG were prepared in the presence or absence of ATP. After incubation at 30°C for the times indicated, the vesicles were UV-irradiated. Cross-linking was detected as in Fig. 1B with antibodies to LPS and LptC. (**B**) LPS is released to LptA in an LptBFGC-, time-, and ATP-dependent manner. RSO membrane vesicles were prepared from wild-type cells or cells overexpressing LptC, LptBFG, or LptBFGC with or without added ATP. Purified LptA(I36*p*BPA) or LptA(H37*p*BPA) was added to these vesicles, incubated at 30°C for the times indicated, and then UV-irradiated. (**C**) Model of the stacked crystal structures of LptC (green) and LptA (purple). Residues Ile[36] and His[37] in LptA, which interact with LPS and LptC, respectively, are depicted as stick structures. (**D**) Periplasmic bridge components properly assemble in vitro. Purified LptA(I36*p*BPA) or LptA(H37*p*BPA) was added to LptC- or LptBFGC-enriched RSO membrane vesicles. Samples were incubated at 30°C for the times indicated, and then UV-irradiated. LptA(H37*p*BPA) cross-linked to LptC in an ATP-, time-, and LptBFG-independent manner.

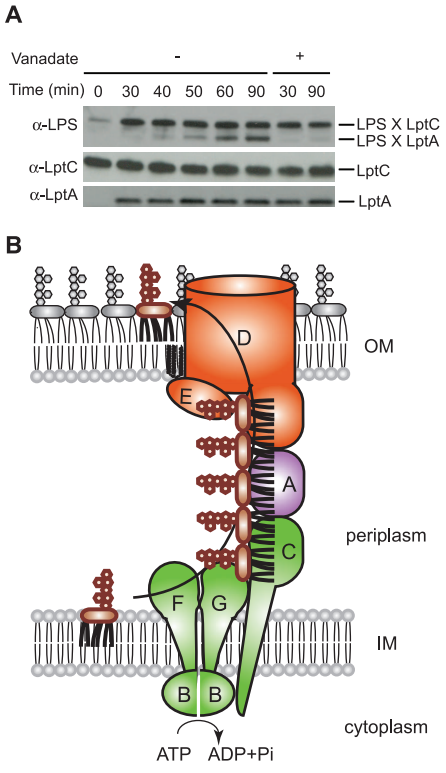

Fig. 4. LPS is pushed in a continuous stream through the Lpt bridge. (**A**) Inhibition of ATP hydrolysis inhibits transfer of LPS from LptC to LptA. RSO membrane vesicles overexpressing LptBFG-LptC (T47*p*BPA) were incubated at 30°C for 30 min in the presence of ATP to accumulate LPS in LptC. Purified LptA(I36*p*BPA) was then added with or without vanadate, and the samples were incubated for an additional 60 min. Cross-linking was detected as in Fig. 1B with antibodies to LPS, LptC, and LptA. (**B**) Model of LPS biogenesis. LptBFG extracts LPS from the inner membrane and transports it to LptC using ATP hydrolysis energy. ATP hydrolysis is used again to push LPS from LptC to LptA.

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Supplementary Materials

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Materials and Methods
Fig. S1
Tables S1 and S2

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Direct Observation of Stalled Fork Restart via Fork Regression in the T4 Replication System

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The restart of a stalled replication fork is a major challenge for DNA replication. Depending on the nature of the damage, different repair processes might be triggered; one is template switching, which is a bypass of a leading-strand lesion via fork regression. Using magnetic tweezers to study the T4 bacteriophage enzymes, we have reproduced in vitro the complete process of template switching. We show that the UvsW DNA helicase in cooperation with the T4 holoenzyme can overcome leading-strand lesion damage by a pseudostochastic process, periodically forming and migrating a four-way Holliday junction. The initiation of the repair process requires partial replisome disassembly via the departure of the replicative helicase. The results support the role of fork regression pathways in DNA repair.

DNA damage causes a replication fork to stall or collapse and is responsible for illegitimate recombination and cellular dysfunction (*1*, *2*). Although damage in the lagging strand is less likely to block fork progression, damage in the leading strand is a particular challenge because of the continuous nature of the leading-strand synthesis. The fork regression pathway is one means to overcome leading-strand lesions, as generally described in terms of four steps (fig. S1): (i) replisome disassembly; (ii) stalled fork regression to form a Holliday junction (HJ) structure (also called a chicken-foot structure); (iii) either lesion excision repair or template switching (*3*, *4*), which is polymerase extension of the 3′ end of the leading strand, now annealed to an intact template, downstream of the lesion (referred here as lesion bypass); and (iv) restoration of the fork and reloading of the replisome (*2*). However, the details of such a pathway and the biological role of regressed forks in vivo are poorly understood (*2*, *5*).

The bacteriophage T4 lacks translesion polymerases and, unlike *Escherichia coli*, cannot reinitiate synthesis by repriming the leading strand (*6*, *7*). Hence, the T4 phage may exclusively use

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