The Lipid A 1-Phosphatase, LpxE, Functionally Connects Multiple Layers of Bacterial Envelope Biogenesis

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ABSTRACT Although distinct lipid phosphatases are thought to be required for processing lipid A (component of the outer leaflet of the outer membrane), glycerophospholipid (component of the inner membrane and the inner leaflet of the outer membrane), and undecaprenyl pyrophosphate (C55-PP; precursors of peptidoglycan and O antigens of lipopolysaccharide) in Gram-negative bacteria, we report that the lipid A 1-phosphatases, LpxEs, functionally connect multiple layers of cell envelope biogenesis in Gram-negative bacteria. We found that Aquifex aeolicus LpxE structurally resembles YodM in Bacillus subtilis, a phosphatase for phosphatidylglycerol phosphate (PGP) with a weak in vitro activity on C55-PP, and rescues Escherichia coli deficient in PGP and C55-PP phosphatase activities; deletion of lpxE in Francisella novicida reduces the MIC value of bacitracin, indicating a significant contribution of LpxE to the native bacterial C55-PP phosphatase activity. Suppression of plasmid-borne lpxE in F. novicida deficient in chromosomally encoded C55-PP phosphatase activities results in cell enlargement, loss of O-antigen repeats of lipopolysaccharide, and ultimately cell death. These discoveries implicate LpxE as the first example of a multifunctional regulatory enzyme that orchestrates lipid A modification, O-antigen production, and peptidoglycan biogenesis to remodel multiple layers of the Gram-negative bacterial envelope.

IMPORTANCE Dephosphorylation of the lipid A 1-phosphate by LpxE in Gram-negative bacteria plays important roles in antibiotic resistance, bacterial virulence, and modulation of the host immune system. Our results demonstrate that in addition to removing the 1-phosphate from lipid A, LpxEs also dephosphorylate undecaprenyl pyrophosphate, an important metabolite for the synthesis of the essential envelope components, peptidoglycan and O-antigen. Therefore, LpxEs participate in multiple layers of biogenesis of the Gram-negative bacterial envelope and increase antibiotic resistance. This discovery marks an important step toward understanding the regulation and biogenesis of the Gram-negative bacterial envelope.

KEYWORDS bacterial cell envelope biogenesis, lipid A 1-phosphate phosphatase, phosphatidylglycerol phosphate phosphatase, type 2 phosphatidic acid phosphatase (PAP2) superfamily, undecaprenyl pyrophosphate phosphatase
at the outer leaflet of the outer membrane through the hydrophobic lipid A moiety. As peptidoglycan, phospholipids, and LPS are synthesized through distinct pathways, how Gram-negative bacteria orchestrate the biogenesis and remodeling across three layers of the cell envelope for optimal bacterial growth and virulence remains incompletely understood.

As the major lipid species coating the outer surface of Gram-negative bacteria, lipid A is the predominant signaling molecule that is detected by the mammalian Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD-2) innate immune receptor (1) and caspase-4/-5/-11 (2) to trigger the host innate immune response to bacterial infection. With few exceptions, Gram-negative bacteria constitutively synthesize the 1,4'-bisphosphorylated tetra-acyl-lipid A intermediate, 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo)-linked lipid IVα (Kdo2-lipid IVα), via the action of seven conserved enzymes in the Raetz pathway (3) (see Fig. S1A in the supplemental material), which are essential to nearly all Gram-negative bacteria and are attractive targets for novel antibiotics (4–6). Gram-negative bacteria additionally harbor modification enzymes that further process the Kdo2-lipid IVα intermediate to generate unique lipid A molecules in each bacterial species to adapt to environmental changes and evade the host immune response (7). For example, the lipid A 1-phosphate is a key determinant for lipid A recognition by the mammalian TLR4/MD-2 innate immune receptor (8). Removal of the lipid A 1-phosphate by the membrane-embedded phosphatase LpxE strongly protects bacteria against host cationic peptides and the last-resort antibiotic colistin (9), significantly dampens the host innate immune response, and dramatically increases colonization and survival of Helicobacter pylori in the gastric mucosa (10).

In order to gain molecular insights into the structure and function of the lipid A 1-phosphatase LpxE, we identified the previously uncharacterized gene aq_1706 from Aquifex aeolicus as the gene for the thermophilic LpxE enzyme (LpxEAA). Our structural analysis of LpxEAA shows distinct features between LpxEAA and Escherichia coli PgpB (PgpBEC) enzymes but reveals a surprising structural similarity to YodM, a phosphatase of phosphatidylglycerol phosphate (PGP) in the Gram-positive bacterium Bacillus subtilis with a weak in vitro activity on undecaprenyl pyrophosphate (C55-PP). Consistent with our structural analysis, we found that LpxEAA possesses substantial in vitro activities toward Kdo2-lipid A/lipid IVα, C55-PP, and PGP and complements E. coli strains deficient in C55-PP phosphatase and PGP phosphatase activities. In addition to the LpxE enzyme from A. aeolicus, distant LpxE orthologs from Francisella, Helicobacter, and Rhizobium also complement E. coli strains deficient in the C55-PP phosphatase activity, supporting the notion that the multifunctional lipid phosphatase activity is a general feature of LpxE enzymes. Significantly, deletion of the native lpxE gene sensitizes Francisella novicida to bacitracin, an antibiotic that sequesters C55-PP to disrupt peptidoglycan synthesis; furthermore, suppression of plasmid-encoded lpxE in the F. novicida strain deficient in the endogenous C55-PP phosphatase activity results in noticeable changes in cell morphology, profound reduction of O-antigen repeats in LPS, and loss of cell viability. Taken together, these observations reveal a previously unappreciated contribution of LpxE to peptidoglycan biogenesis and LPS O-antigen modification beyond its well-recognized role as the lipid A 1-phosphatase to orchestrate the remodeling of multiple layers of the Gram-negative bacterial envelope to respond to environmental changes, evade host immune surveillance, and promote bacterial viability and virulence.

RESULTS

A distant ortholog of LpxEFN in A. aeolicus. LpxE is a member of the lipid phosphatase/phosphotransferase (LPT) family, a well-distributed family of lipid-processing enzymes also known as the integral transmembrane branch of the type II phosphatidic acid phosphatase (PAP2) superfamily (11, 12). This family is characterized by a conserved tripartite active site motif of KX,RP—SGH—SRX,HX,D and activity independent of Mg2+ or other cations (13). The LPT family includes enzymes responsible for processing several types of lipids in Gram-negative bacteria, including the
membrane-embedded PgpB, which dephosphorylates PGP and C_{SS}-PP (14) (Fig. S2). Even though PgpB and LpxE are both members of the LPT family, they have been reported to have distinct substrate specificities: PgpB is unable to utilize lipid A as a substrate (15), whereas purified LpxE from *Rhizobium leguminosarum* (LpxERL) utilizes PGP ∼1,000 times less efficiently than lipid A species as a substrate *in vitro* (16).

In order to gain a molecular understanding of the LpxE structure and function, we searched for a thermophilic LpxE enzyme from *Aquificae* to facilitate structural analysis. The lipid A of *Aquifex pyrophilus* LPS contains D-galacturonic acid in place of phosphates at the 1- and 4′-positions (17) (Fig. S1B). As the 1,4′-bisphosphorylated lipid IV_{A} is a common lipid A intermediate before further modification (18, 19) and as *Aquificae* has the conserved biosynthetic enzymes to make 1,4′-bisphosphorylated lipid IV_{A}, incorporation of the D-galacturonic acid moiety requires the removal of 1-phosphate from lipid A, indicating the presence of the lipid A 1-phosphatase activity in *Aquificae*. Such a rationale led us to search for the gene responsible for the lipid A 1-phosphatase activity in *A. aeolicus* VF5, as no lipid A 1-phosphatase has been reported in any *Aquificae* species.

A Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST) (20) search revealed a distant ortholog of *F. novicida* LpxE (LpxE_{PN}) (15), Aq_1706 (E value, 0.81; sequence identity, 13.84%), in the genome of *A. aeolicus* VF5. Aq_1706 shares little sequence identity with other LpxE enzymes (sequence identities of Aq_1706 with LpxE of *Helicobacter* and *Rhizobium* are 16.58% and 14.57%, respectively), except for the well-conserved tripartite active-site motif of KX_{6}RP---PSGH---SRX_{5}HX_{3}D (Fig. S3). In order to determine if *aq_1706* encodes the lipid A 1-phosphatase activity *in vivo*, we overexpressed Aq_1706 in the heptose transferase-deficient *E. coli* strain WBB06, which produces Kdo_{2}-lipid A instead of full-length LPS, to facilitate mass spectrometry analysis of lipid A modifications (21). Since *E. coli* does not encode LpxE activity, mass spectrometry analysis of the extracted lipids showed normal lipid A containing 1-phosphate with an m/z of 1,117.633 for the [M-2H]^{2-} ion species (calculated m/z, 1,117.661 for the exact mass of 2,237.336 of Kdo_{2}-lipid A) from *E. coli* cells expressing a control vector; in contrast, overexpression of Aq_1706 in *E. coli* led to the disappearance of the intact lipid A species and significant accumulation of lipid A molecules lacking the 1-phosphate group, with an m/z of 1,077.647 for the [M-2H]^{2-} ion species (calculated m/z, 1,077.678 for the exact mass of 2,157.370 of 1-dephospho Kdo_{2}-lipid A), consistent with the anticipated lipid A 1-phosphatase activity (Fig. 1A). In order to verify that the loss of phosphate occurred at the 1-position, but not at the 4′-position, we further tested the ability of LpxE to dephosphorylate 4′-32P-labeled Kdo_{2}-lipid A, which was previously shown to be an efficient substrate for LpxE enzymes with specific activity comparable to that for the substrate Kdo_{2}-lipid A (16). We found that treatment of Kdo_{2}-[4′-32P] lipid IV_{A} with membrane extracts from *E. coli* overexpressing Aq_1706, but not those carrying a control vector, resulted in time-dependent reduction of the Kdo_{2}-lipid IV_{A} band and accumulation of an upper-shifted band on the thin-layer chromatography (TLC) plate (Fig. 1B), reflecting the removal of 1-phosphate but retention of the 32P-labeled 4′-phosphate group. Taken together, these observations verify *aq_1706* in *A. aeolicus* as the gene that encodes the thermophilic lipid A 1-phosphatase LpxE (LpxE_{AA}).

**Structural analysis of LpxE_{AA} reveals a striking similarity to YodM in *B. subtilis***. After verifying the lipid A 1-phosphatase activity of LpxE_{AA}, we cloned and purified LpxE_{AA}. Consistent with the TMHMM analysis (http://www.cbs.dtu.dk/services/TMHMM/), high-yield expression of LpxE_{AA} was achieved in a maltose-binding protein (MBP) fusion construct containing an N-terminal PelB secretion signal (22), suggesting that the N terminus of LpxE_{AA} is located at the periplasmic side of the inner membrane. The crystal structure of LpxE_{AA} containing an I63M mutation was determined at 2.38 Å (Fig. 2A; statistics shown in Table S1). The selenomethionine substitution of the nonconserved I63 residue (I63M) was designed to enhance the selenium single anomalous dispersion (Se-SAD) signal for *de novo* phasing. The overall structure of LpxE_{AA} contains seven α-helices, including an N-terminal amphipathic helix lying at
the periplasmic surface of the inner membrane and five tightly packed transmembrane helices (α3 to α7). Apart from α2, which originates from the periplasmic surface and penetrates halfway across the inner membrane at an ~45° angle and immediately connects to transmembrane helix α3, the remaining helices are oriented largely in parallel or antiparallel with each other and perpendicularly to the membrane plane. Looking from the periplasmic surface, helix 5 (α5) is located at the center, which is surrounded by α2, α3, α4, α7, and α6 in a counterclockwise fashion (Fig. 2B).

The active site of LpxE is located at the periplasmic surface of the inner membrane and is defined by conserved motifs specific to the PAP2 enzymes (K73X6R80P---R137X5H143X3D147) located at the C-terminal end of α4, the α4-α5 loop, α6, the α6-α7 loop, and the N terminus of α7 (Fig. 2C). Fortuitously, a sulfate molecule is found in the active site, which is a structural analog of the 1-phosphate group of lipid A. The catalytically important H143 is located 3.3 Å away from the sulfur atom of the sulfate group, ready to carry out inline attack to remove the phosphate group of the lipid substrate. D147, the last residue of the R137X_H143X_D147 motif, forms a hydrogen bond with H143. Although the corresponding aspartate residue is found in most LpxE enzymes (Fig. S3), it is absent in the LpxE ortholog from H. pylori (LpxE_Hp), suggesting that it is not absolutely required for catalysis. The first three residues of the PSGH motif are conserved in LpxE_Aa, with the central serine
residue (S101) serving as a helix cap to stabilize helix α5, but the histidine residue is replaced with an aspartate residue in LpxEAA (Fig. 2C).

The LpxEAA structure shows noticeable conformational discrepancy with the previously reported structures of PgpBEC (PDB codes 4PX7 and 5JWY) (23, 24), another PAP2 family enzyme, with overall backbone root mean square deviations (RMSDs) of ∼4.5 Å (Fig. S4); surprisingly, LpxEAA is structurally similar to the recently reported YodM in B. subtilis (PDB code 5JKI) (25), a PGP phosphatase with a weak in vitro activity on C55-PP, with an overall backbone RMSD of 1.2 Å (Fig. 2D). The major differences of these two enzymes are the absence of an N-terminal transmembrane helix in LpxEAA in comparison with YodM, a longer α4-α5 loop in LpxEAA, and a significant conformational variation of the α4-α5 loop surrounding the active site.

LpxEAA is a trifunctional lipid phosphatase in vitro and functionally complements E. coli mutants deficient in C55-PP or PGP phosphatase activities. Surprised
by the structural similarity between LpxEAA and YodMBS, we asked whether LpxEAA could function as a C55-PP and PGP phosphatase. To address this question, we compared the specific activities of purified LpxEAA toward Kdo2-lipid A, PGP, and C55-PP using the malachite green assay to detect the release of inorganic phosphate. As expected, LpxEAA efficiently catalyzed the hydrolysis of 1-phosphate from Kdo2-lipid A, with a specific activity of 2.04 ± 0.46 μmol/mg/min. Moreover, LpxEAA catalyzed C55-PP more efficiently than it catalyzed Kdo2-lipid A, with a specific activity of 3.58 ± 0.47 μmol/mg/min—a value that is ~1.8-fold higher than that toward Kdo2-lipid A. Finally, LpxEAA also displayed significant activity toward PGP, with a specific activity of 0.75 ± 0.11 μmol/mg/min, ~40% of its activity toward Kdo2-lipid A (Table 1). Taken together, our biochemical assays validate LpxEAA as a trifunctional LPT enzyme that efficiently dephosphorylates chemically diverse Kdo2-lipid A (glycolipids), PGP (phosphoglycerol lipid), and C55-PP (isoprenyl lipid) in vitro.

In order to obtain further evidence of the trifunctional role of LpxEAA in cells, we examined whether LpxEAA could functionally rescue lethal E. coli mutants lacking C55-PP phosphatase or PGP phosphatase activities. E. coli contains four C55-PP phosphatases, BacA, PgpB, YbjG, and LpxT. A deletion mutant, ΔybJG ΔbacA Δp gpB::kan, in E. coli is lethal unless rescued by a plasmid expressing BacA, PgpB, or YbjG (26). To examine if LpxEAA could function as a C55-PP phosphatase in cells, we set up complementation of the lethal ΔybJG Δp gpB ΔbacA::kan E. coli mutant carrying lpxEAA on a low-copy-number, temperature-sensitive pMAK705 vector (pMAK-lpxEAA). The E. coli bacA gene, encoding the C55-PP phosphatase, was used as the positive control (pMAK-bacAEC). We found that overexpression of LpxEAA and BacAEC from pMAK705-derived plasmids complemented the lethal phenotype of the ΔybJG Δp gpB ΔbacA::kan triple knockout in E. coli on an LB agar plate at 30°C; such a complementation effect was lost when cells were grown at 42°C, consistent with the loss of the temperature-sensitive pMAK705 plasmid encoding LpxEAA or BacAEC and confirming that LpxEAA functionally complements the loss of C55-PP phosphatase activity in E. coli (Fig. 3A).

We similarly tested whether LpxEAA functionally complements the loss of PGP phosphatase activity in E. coli. E. coli has three PGP phosphatases, PgpA, PgpB, and PgpC (27). A Δp gpP A Δp gpB Δp gpC::kan triple-knockout mutant is lethal unless it is rescued by a plasmid harboring an active PGP phosphatase (27). Overexpression of LpxEAA or the positive control PgpAEC from the temperature-sensitive pMAK705 plasmid supported the growth of the Δp gpP A Δp gpB Δp gpC::kan triple-knockout mutant strains at 30°C but not at 42°C. In contrast, the control strain (W3110/pMAK705) grew well at both temperatures (Fig. 3B). These observations confirm that LpxEAA is a functional PGP phosphatase in E. coli.

While the pMAK705 vector-encoded LpxEAA complemented E. coli triple knockouts lacking C55-PP phosphatase or PGP phosphatase activities, pMAK705 has a higher copy number (pSC101 origin, ~5 copies/cell) than that of the chromosome in E. coli (single copy/cell). In order to mitigate the concern that the observed genetic complementation was caused by multiple copies of the lpxEAA gene, we replaced the pgpB gene in the chromosome of E. coli (BW25113) ΔybJG ΔbacA with a gene cassette (PPL-lpxEAA-FRT-kan-FRT) containing lpxEAA and a kanamycin resistance gene under the control of the PPL promoter (28). The resulting E. coli strain (E. coli BW25113 ΔybJG ΔbacA Δp gpP::PPL-lpxEAA-FRT-kan-FRT) grew on an LB agar plate, and the proper knockouts of bacA, ybjG, and p gpP were verified by PCR (Fig. 3C), confirming that the chromosomal copy of lpxEAA complemented the loss of C55-PP phosphatase activity. Using a similar approach,
FIG 3 LpxEAA complements E. coli strains deficient in C55-PP phosphatase or PGP phosphatase activities. (A) Complementation of the C55-PP phosphatase-deficient E. coli strain (BW25113 ΔybjG ΔpgpB ΔbacA::kan) by the temperature-sensitive pMAK705 plasmid harboring bacAEC (positive control, pMAK-bacAEC) or lpxEAA (pMAK-lpxEAA). WT E. coli cells carrying pMAK705 or C55-PP phosphatase-deficient E. coli cells carrying pMAK-bacAEC or pMAK-lpxEAA were grown at 30°C or 42°C. From left to right are spots of 10-fold serial dilutions from 10^2 to 10^5. (B) Complementation of the PGP phosphatase-deficient E. coli strain (W3110 ΔpgpA ΔpgpB ΔpgpC::kan) by the temperature-sensitive pMAK705 plasmid harboring pgpAEC (positive control, pMAK-pgpAEC) or lpxEAA (pMAK-lpxEAA). WT E. coli cells carrying pMAK705 or PGP phosphatase-deficient E. coli cells carrying pMAK-pgpAEC or pMAK-lpxEAA were grown at 30°C or 42°C. From left to right are spots of 10-fold serial dilutions from 10^2 to 10^5. (C) Chromosomal complementation of C55-PP phosphatase activity-deficient E. coli with lpxEAA. The left, middle, and right images show the construction of different E. coli C55-PP phosphatase gene deletion mutants, the growth of WT E. coli cells and C55-PP phosphatase-deficient cells complemented by a chromosomal copy of lpxEAA, and PCR verification of ybjG, bacA, and pgpB knockouts of the target mutant strain, respectively. (D) Chromosomal complementation of PGP phosphatase activity-deficient E. coli with lpxEAA. The left, middle, and right images show the construction of different E. coli PGP phosphatase gene deletion mutants, the growth of WT E. coli cells and PGP phosphatase-deficient cells complemented by a chromosomal copy of lpxEAA, and PCR verification of pgpA, pgpC, and pgpB knockouts of the target mutant strain, respectively. Since the expected sizes of pgpB (1,106 bp) and pgpB::PL-lpxEAA (1,093 bp) are similar using primers flanking pgpB in the final strain, the knockout of pgpB was established by also verifying the PCR result of the mother strain (strain 5: W3110 ΔpgpA ΔpgpB::PL-lpxEAA-kan-frt).
we also replaced the pgpB gene of E. coli (W3110) ΔpgpA with P_lpxEAA-FRT-kan-FRT, removed the kanamycin resistance cassette (29), and then knocked out pgpC. The resulting strain (E. coli W3110 ΔpgpA ΔpgpB::P_lpxEAA ΔpgpC::kan) also grew on an LB agar plate, and knockouts of pgpA, pgpB, and pgpC were verified by PCR (Fig. 3D), confirming that the chromosomal copy of lpxEAA similarly complemented the loss of PGP phosphatase activity.

Altogether, the substantial phosphatase activities of LpxEAA toward Kdo2-lipid A, C55-PP, and PGP in vitro and its ability to complement the loss of C55-PP and PGP phosphatase activities in E. coli—both via the plasmid-borne gene and via chromosomal knock-in—strongly support the multifunctionality of LpxEAA in Gram-negative bacterial envelope biogenesis.

LpxE_FN is a bifunctional lipid phosphatase in vitro and functionally complements an E. coli mutant deficient in the C55-PP phosphatase activity. Despite the intriguing observation of the multifunctionality of LpxEAA, it is challenging to establish the biological consequence in its native host due to the difficulty of culturing and genetic manipulation of A. aeolicus. Therefore, we asked if other LpxE enzymes from genetically tractable bacteria similarly display multifunctional lipid phosphatase activities. In order to answer this question, we chose LpxEFN, a distant ortholog of LpxEAA, for further characterization. The ability of LpxE_FN to dephosphorylate lipid A at the 1-position was previously reported (15), but its activity toward other lipid substrates has not been thoroughly investigated. We first conducted similar complementation experiments using E. coli strains deficient in either the C55-PP phosphatase activity or PGP phosphatase activity carrying the temperature-sensitive pMAK-lpxE_FN. We found that LpxE_FN complemented the loss of C55-PP phosphatase activity of E. coli (ΔybjG ΔpgpB ΔbacA::kan) at 30°C but not at 42°C, indicating that LpxE_FN is a functional C55-PP phosphatase in E. coli (Fig. 4A). However, we were unable to complement E. coli deficient in the PGP activity (ΔppgA ΔppgB ΔppgC::kan) with a plasmid encoding LpxE_FN (pMAK-lpxE_FN). Consistently, we found that purified LpxE_FN displayed significant phosphatase activity toward both Kdo2-lipid A and C55-PP and processed these two substrates with similar efficiencies (specific activities of 3.25 ± 0.21 μmol/mg/min for Kdo2-lipid A and 2.99 ± 0.45 μmol/mg/min for C55-PP), but its activity toward PGP was ~100-fold lower (specific activity of 0.038 ± 0.009 μmol/mg/min) (Table 1), confirming that LpxE_FN is a bifunctional lipid phosphatase.

F. novicida harbors two C55-PP phosphatases: LpxE_FN and FTN_1552. It is important to note that the lipid A 1-phosphatase activity is not essential in bacteria but the C55-PP phosphatase activity is. Prior to this study, no enzyme encoding the C55-PP phosphatase activity had been identified in F. novicida. As the transposon mutant of lpxE_FN is not lethal in F. novicida (30), we reasoned that there must exist another enzyme encoding the C55-PP phosphatase activity in F. novicida. By searching for F. novicida proteins homologous to E. coli enzymes containing C55-PP phosphatase activity (i.e., BacAEC, YbjGEC, PgpBEC, and LpxTEC) using PSI-BLAST (20), we have identified a PAP2 family protein of unknown function, FTN_1552, as a potential candidate of the C55-PP phosphatase (PSI-BLAST of PgpBEC: E value of 0.003 and sequence identity of 16.47%). We found that the temperature-sensitive pMAK705 vector harboring ftn_1552 complemented the E. coli strain deficient in C55-PP phosphatase activity (ΔybjG ΔppgB ΔbacA::kan), confirming ftn_1552 as the gene encoding the C55-PP phosphatase activity (Fig. 4A). FTN_1552 was subsequently renamed UppP_FN. Purified UppP_FN appears to be a specific enzyme for C55-PP, with a specific activity of 22.71 ± 2.62 μmol/mg/min, and displays little activity toward Kdo2-lipid A and PGP (specific activities of 0.010 ± 0.005 μmol/mg/min and 0.031 ± 0.007 μmol/mg/min, respectively [Table 1]). Importantly, while F. novicida strains containing a chromosomal deletion of either lpxE_FN or uppP_FN were viable, we were unable to generate F. novicida strains containing both deletions (ΔlpxE_FN ΔuppP_FN) in the chromosome (Fig. 4B and C). However, F. novicida cells were viable in the ΔuppP_FN background when lpxE_FN was replaced with lpxE_AA (Fig. 4B and C). Furthermore, when F. novicida was first transformed with a
plasmid (pEDL17) bearing \(\text{lpxEFN}\) under the control of an anhydrotetracycline (aTc) promoter, we were also able to obtain viable \(\text{F. novicida}\) colonies containing chromosomal deletions of both \(\text{lpxEFN}\) and \(\text{uppPFN}\) (\(\text{U112 } \Delta\text{lpxEFN}::\text{kan}\)). The presence of proper chromosomal deletions of the \(\text{lpxEFN}\) and \(\text{uppPFN}\) genes was verified by PCR (Fig. 4D). As expected, the viability of such a strain depends on the expression of plasmid-encoded LpxEFN: prolonged withdrawal of aTc suppressed the bacterial growth and slowly resulted in cell lysis in culture (Fig. 4D), reinforcing the notion that UpP\text{PFN} and LpxEFN share redundant \(\text{C}_{55}\)-PP phosphatase activities in \(\text{Francisella}\).

\text{LpxEFN functionally connects multiple layers of envelope biogenesis in } \text{F. novicida. After establishing that LpxEFN shares } \text{C}_{55}\text{-PP phosphatase activity with UpP\text{PFN}, we further examined the biological implication of the multifunctional enzymatic activity of LpxEFN in its native host, } \text{F. novicida. We first verified the role of LpxEFN}}
as a lipid A 1-phosphatase. Wild-type (WT) *F. novicida* cells contain both LPS (i.e., core oligosaccharide and O-antigen-modified Kdo-lipid A3 without 1- and 4'-phosphates) and free lipid A species A1 and A2, which do not contain core oligosaccharides/Kdo or O-antigen (lipid A2 differs from lipid A1 in that it has an additional α-linked glucose moiety attached to its 6'-position; also see the schematic lipid A structures of WT *F. novicida* in Fig. 5A) (31). Both lipid A1 and lipid A2 are further modified by FlmK, which transfers galactosamine from C55-P-galactosamine to the 1-phosphate of lipid A (32, 33). As the core oligosaccharide and O-antigen-modified lipid A are inefficiently extracted by the Bligh-Dyer method for mass spectrometry analysis, we examined the effect of ΔlpxEFN in the *F. novicida* strain deficient in the glycosyltransferase activity (ΔpocC), which produces Kdo-lipid A3 (instead of LPS), in addition to lipids A1 and A2 found in the wild-type cells (Fig. 5A). Accumulations of Kdo-(1-phospho)-lipid A3 and Kdo-(galactosamine-1-phospho)-lipid A3, as well as the disappearance of Kdo-lipid A3, were observed in *F. novicida* when lpxEFN was deleted (ΔlpxEFN), confirming the lipid A 1-phosphatase activity of LpxEFN in cells (Fig. 5A and Fig. 5S).

As the C55-PP phosphatase activity of LpxEFN (2.99 ± 0.45 μmol/mg/min) is only ~7-fold smaller than that of UppPFN (22.71 ± 2.62 μmol/mg/min), we asked whether LpxEFN could functionally contribute to the bacterial envelope biogenesis beyond lipid A modification at the 1-phosphate position. We first compared the sensitivities of the wild-type *F. novicida* strain (U112) and mutant strains containing either the lpxE or uppP deletion to bacitracin, an antibiotic sequestering C55-PP. We found that while the loss of uppP in *F. novicida* generated an 8.5-fold drop of MIC in comparison with that of the WT strain (0.5 μM versus 4.25 μM), as expected, the loss of lpxE also resulted in ~1.7-fold drop of the MIC of bacitracin (2.5 μM for *F. novicida* ΔlpxE) (Fig. 5B), implicating a functional role of LpxEFN in the recycling of C55-PP.

In order to isolate the biological effect of LpxEFN, we utilized the *F. novicida* strain containing chromosomal deletions of both uppP and lpxE, which is complemented by a plasmid carrying lpxEFN under the control of an aTc promoter. We found that the loss of plasmid-mediated expression of LpxEFN due to withdrawal of aTc in the growth medium resulted in cell enlargement, reflecting defective peptidoglycan biosynthesis (Fig. 5C). Strikingly, while no change of O-antigen repeats was observed in *F. novicida* cells containing the chromosomal deletion of either lpxE or uppP in comparison with WT cells (Fig. 5D), transient suppression of LpxE led to a dramatic reduction of the LPS O-antigen repeats, including both high- and low-repeat species (Fig. 5E) (34), suggesting a contribution of LpxE to the O-antigen biogenesis. These observations are consistent with the notion that the biosynthesis and transport of peptidoglycan and O-antigen depend on C55-PP, the product of LpxEFN (and UppPFN) activity, and reveal a previously unappreciated function of LpxE in the biogenesis and remodeling of multiple components across the bacterial envelope: peptidoglycan, free lipid A, and the O-antigen repeat of LPS.

**DISCUSSION**

LpxE enzymes are important virulence factors that promote bacterial survival, fitness, and pathogenicity. In *H. pylori* and *Rhizobium etli* CE3, the chromosomal knockout of lpxE resulted in increased susceptibility to positively charged antimicrobial peptides such as polymyxin B and colistin (9, 35), presumably due to the retention of 1-phosphate of lipid A. Previous studies showed that *Rhizobium* LpxE displays over a 1,000-fold preference of Kdo2-lipid A/lipid IVA over PGP; therefore, LpxE has been regarded as a highly specific monofunctional enzyme whose sole activity is to remove the 1-phosphate from lipid A. In this study, based on the striking structural similarity between LpxE_Aa and YodM_Bs, a PGP phosphatase with a weak in vitro activity on C55-PP phosphatase, we discovered that LpxE is a multifunctional lipid phosphatase. The LpxE enzyme from *A. aelius* displays significant activities toward Kdo2-lipid A/lipid IVA, C55-PP and PGP and functionally complements *E. coli* strains deficient in C55-PP or PGP phosphatase activities. Likewise, the LpxE enzyme from *F. novicida* is a dual-function enzyme that processes Kdo2-lipid A and C55-PP with similar efficiencies. Strikingly,
deletion of \textit{lpxEFN} in its native host \textit{F. novicida} resulted in accumulation of phosphorylated lipid A3 species and increased sensitivity to bacitracin; in the \(\Delta uppP\) and \(\Delta lpxE\) double-knockout mutant \textit{F. novicida}, suppression of \textit{lpxEFN} expression from the plasmid resulted in cell deformation due to defective peptidogly-
can biosynthesis and the loss of O-antigen repeats in LPS associated with reduced O-antigen transport, both of which are critically dependent on the recycling of C\textsubscript{55}-PP to C\textsubscript{55}-P. Taken together, these results show that LpxE enzymes from \textit{A. aeolicus} and \textit{F. novicida} functionally connect multiple layers of bacterial envelope biogenesis and remodeling. Such multiple functional roles are not unique to LpxE enzymes from \textit{A. aeolicus} and \textit{F. novicida}: we found that LpxE enzymes from \textit{H. pylori} and \textit{R. leguminosarum} also complemented \textit{E. coli} deficient in C\textsubscript{55}-PP phosphatase activities (Fig. S6), suggesting that these LpxE enzymes can similarly process Kdo\textsubscript{2}-lipid A and C\textsubscript{55}-PP to synchronize lipid A modification with peptidoglycan biosynthesis and O-antigen modification of LPS.

It is appropriate to ask why LpxE has evolved into a multifunctional enzyme. There are several potential explanations. First, it is conceivable that the peptidoglycan biosynthesis is such an essential process that multiple enzymes, including LpxE, are employed as the backup enzymes for the C\textsubscript{55}-PP phosphatase-mediated recycling reaction for peptidoglycan charging and biosynthesis. Second, it is possible that LpxE from \textit{Aquifex} species represents an ancestral lipid phosphatase, which, although primitive, is sufficient to conduct all lipid phosphatase activities to support the bacterial envelope biogenesis and remodeling, while other LPT family of lipid phosphatases, such as the PGP phosphatase, evolved later as specialized, highly efficient enzymes. Third, it is also likely that LpxE evolved as a multifunctional enzyme to coordinate lipid A modification and the biogenesis of other layers of bacterial envelope. As 1,4′-bisphosphorylated lipid A chelates metal ions to form a fortified layer for bacterial protection, removal of the 1-phosphate could weaken the lipid A layer and increase membrane permeability. It is conceivable that the weakened lipid A layer is compensated by the elevated peptidoglycan biosynthesis and enhanced O-antigen decoration of LPS. Thus, bestowing LpxE with the multifunctionality toward Kdo\textsubscript{2}-lipid A and C\textsubscript{55}-PP (and, in the case of \textit{A. aeolicus}, PGP) enables LpxE to orchestrate lipid A modification with bacterial envelope remodeling at multiple layers (Fig. 6) in order to promote the optimal bacterial growth and enhance bacterial survival in nature and the human host.

The Gram-negative bacterial envelope contains three layers. How Gram-negative bacteria coordinate the biogenesis and remodeling of different layers of the bacterial envelope has remained an area of active investigation. Our study has revealed the first biological evidence of a multifunctional enzyme, LpxE in \textit{F. novicida}, that natively couples lipid A 1-dephosphorylation with C\textsubscript{55}-PP recycling to enhance peptidoglycan biogenesis and O-antigen decoration of LPS, promote cell viability against antimicrobial
peptides, evade host immune surveillance, and ultimately support bacterial pathogenesis. We suggest that such a multifunctional role represents a common but previously unappreciated mechanism for Gram-negative bacteria to coordinate bacterial envelope biogenesis across different layers.

**MATERIALS AND METHODS**

Data collection and refinement statistics of LpxE are listed in Table S1. All strains and plasmids used in this work are listed in Tables S2 and S3, respectively. Plasmid and strain constructions and growth conditions are described in the supplemental material in detail.

Extraction of lipid A species, TLC and mass spectrometry analyses of lipid A species, and assay conditions are described in the Supplementary Methods section of the supplemental material.

Characterizations of F. novicida U112 mutants are described in the Supplementary Methods section of the supplemental material.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00886-19.

TEXT S1, DOCX file, 0.1 MB.

FIG S1, TIFF file, 2.8 MB.

FIG S2, TIFF file, 2.8 MB.

FIG S3, TIFF file, 2.9 MB.

FIG S4, TIFF file, 2.8 MB.

FIG S5, TIFF file, 2.8 MB.

FIG S6, TIFF file, 2.2 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.02 MB.

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