Supporting Information

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SI Materials and Methods

LPS Purification and Lipid A Isolation. *Fn* and *Escherichia coli* LPS were extracted using a hot phenol/water extraction method as described (1). Further treatment of LPS with RNase A, DNase I, and proteinase K ensured removal of contaminating nucleic acids and proteins (2). Subsequently, LPS samples were additionally subjected to Folch and Vogel extractions to remove contaminating phospholipids and TLR-2 agonist-contaminating proteins, respectively (3, 4). Lipid A was isolated after hydrolysis in 1% SDS (wt/vol) at pH 4.5 as described (5). Briefly, 500 μL of 1% SDS (wt/vol) in 10 mM sodium acetate, pH 4.5, was added to a lyophilized sample. Samples were incubated at 100 °C for 1 h and lyophilized. The dried pellets were washed in 100 μL of water and 1 mL of acidified ethanol [100 μL 4N HCl in 20 mL 95% ethanol (vol/vol)]. Samples were centrifuged at 5,000 × *g* for 5 min. The lipid A pellet was further washed (three times) in 1 mL of 95% ethanol. The entire series of washes was repeated twice. Samples were resuspended in 500 μL of water, frozen on dry ice, and lyophilized.

One-Step Total Lipids Isolation Method. Lyophilized cells (100 ng–10 μg) were suspended in 100 μL chloroform:methanol (2:1, vol/vol), vortexed for 1 min, and centrifuged at 5,000 × *g* for 1 min. The resulting supernatant was collected and spotted (1 μL) directly onto the MALDI sample plate followed by 1 μL matrix for matrix-assisted laser desorption ionization (MALDI)-TOF/TOF MS analysis. The supernatant was dried under a stream of nitrogen (6).

Mass Spectrometry and Gas Chromatography Procedures. Lipid A samples were prepared for analysis at 1.0 mg/mL in chloroform:methanol (1:1, v:v). The sample solution was first mixed 1:1 with a matrix solution, which consisted of 10 mg/mL of 5-Chloro-2-mercaptobenzothiazole (CMBT) in chloroform:methanol:water (4:4:1, v:v:v). Samples were spotted onto the stainless steel 384 target and allowed to air dry before insertion into the mass spectrometer. Analyses were performed on a Kratos Axima QIT (Shimadzu) MALDI quadrupole ion trap time-of-flight (QIT-TOF) mass spectrometer equipped with a 337-nm N2 laser. The instrument was operated in the negative ion mode and calibrated externally using a peptide mixture that consisted of angiotensin II, angiotensin I, neurotensin, ACTH clip (117), and ACTH clip. Argon was used as the collision gas for MS^n. Typically, 1,000–3,000 laser shots were summed for each MS and MS^n analysis.

LPS fatty acids were derivatized to fatty acid methyl esters and analyzed by gas chromatography as described (7, 8). Briefly, LPS fatty acids were derivatized to fatty methyl esters with 2 M methanolic HCl at 90 °C for 18 h (Alltech) and quantified by GC using an HP 5890 series II with a 7673 auto injector. Pentadecanoic acid (10 μg; Sigma) was added as an internal standard.

Construction of *Fn lpxD1*-Null and *lpxD2*-Null Mutants. Two linear deletion fragments of DNA consisting of kanamycin cassette flanked by 1,500 bp of *Fn lpxD1* and *lpxD2* upstream and downstream were generated by overlap PCR. Next, two linear deletion fragments were transformed into *Fn* by chemical transformation, and resistant colonies were selected on Tryptic soy broth supplemented with cysteine (TSB-C) plate containing kanamycin (10 μg/mL). *Francisella novicida* were grown in 100 mL of Chamberlain's medium to A_{600} of 0.5 (9). Cells were harvested by centrifugation and resuspended in 5 mL of transformation buffer. Next, 1 mL of the cell suspension was mixed with 500 ng of the knockout fragment and incubated with shaking (100 rpm at 37 °C) for 30 min, 5 mL of Chamberlain's medium was added, and the mixture was shaken at 37 °C for 3 h at 250 rpm. The cells were harvested and resuspended in 1 mL of TSB-C plate containing kanamycin (10 μg/mL). Genomic DNA was isolated from a kanamycin-resistant transformant, and the replacement of the *lpxD1* and *lpxD2* genes was confirmed by PCR and DNA sequencing.

Cloning LpxD1 and LpxD2 and Complementation Expressing in *Fn*. The *lpxD1* and *lpxD2* coding regions were amplified from genomic *Fn* DNA prepared by PCR with *Pfu* Turbo DNA polymerase (Stratagene). The primers (pMP_*lpxD*1_forward, pMP_*lpxD1*_reverse, pMP_*lpxD*2_forward, pMP_*lpxD2*_reverse) were used for PCR amplification. PCR amplified *lpxD1* and *lpxD2* DNA were cloned into the plasmid pMP822 and pMP831 (10). The resulting plasmids, designed pMP822-*lpxD1*, pMP822-*lpxD2*, pMP831-*lpxD1*, pMP831-*lpxD2*, were introduced into *Fn* cells by cryotransformation, and the resulting bacterial transformants were selected on TSB-C plates at 37 °C containing hygromycin (100 μg/mL).

RNA Isolation and Quantitative PCR. RNA was extracted from midlog phase (OD_{600} ~0.4–0.5) grown *Fn* at 21 °C and 37 °C by the RiboPure-Bacteria kit (Ambion). Five micrograms of total RNA was reverse transcribed to cDNA by SuperScript-II RNase H reverse transcriptase (Invitrogen). Five nanograms of cDNA was used for quantitative PCR with the SYBR green PCR master mixture in the Bio-Rad iCycler. Relative quantification was used to evaluate the expression of chosen genes. All primers were designed to give 200–220 nucleotide amplicons, have a G + C range of 30–50%, and a melting temperature of 58–60 °C. Relative copy numbers (RCN) and expression ratios of selected genes were normalized to the expression of 16s rRNA, and *dnaK*.

LpxD1 and LpxD2 Expression and Purification. Two plasmids (designated pET28a-*lpxD1* and pET28a-*lpxD2*), which encode a fully functional LpxD1 and LpxD2 protein, respectively, modified with an N-terminal 6His tag followed by a one-glycine residue linker and the P2A substitution, was constructed and transformed into *E. coli* Rosetta (DE3)/pLysS (Invitrogen) (11, 12). Expression of LpxD1 and LpxD2 at 37 °C was induced for 3.5 h with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) when the A_{600} of the cells, growing on LB broth supplemented with 100 μg/mL kanamycin, reached 0.5. All subsequent procedures were carried out at 4 °C. Cells from a 3-L culture were harvested by centrifugation at 4,000 × *g*. The pellet was resuspended in 50 mL of buffer A [50 mM Tris chloride, pH 8.0, containing 200 mM sodium chloride, 1 mM DTT, 10% glycerol (vol/vol)], and the cells were again centrifuged at 4,000 × *g*. The pellet was resuspended in 50 mL of buffer A and the cells were lysed by one passage through a French pressure chamber at 18,000 psi. After a 30-min centrifugation at 4,000 × *g*, the cell-free extract was loaded onto a 10-mL Ni-NTA column (Qiagen). After washing with 100 mL of buffer A, LpxD was eluted in one fraction with 50 mL of buffer B containing 200 mM imidazole, and the entire fraction was loaded onto a 318-mL High Load 26/60 Superdex-200 gel filtration column (GE Healthcare), equilibrated in 10 mM Tris chloride, pH 8.0, containing 1 mM DTT and 500 mM NaCl. The protein eluted as a sharp peak consistent with the molecular weight predicted for the LpxD homotrimer. Fractions were pooled and concentrated to 24 mg/mL, as determined by the bicinchoninic acid assay (Bio-Rad). The

protein solution was then passed through a 0.2-μm filter and stored in aliquots at −80 °C.

Cationic Antimicrobial Peptides Sensitivity and Antibiotic Minimum Inhibitory Concentration (MIC) Test. Susceptibility of *lpxD1*-null mutant, *lpxD2*-null mutant, WT *Fn* to polymyxin B was determined using a commercial gradient strip assay (Etest AB; bioMerieux) according to the manufacturer's instructions. The MIC test uses 2× strength TSB-C, 4× strength antibiotic solutions prepared as serial twofold dilutions, and the bacterial strain to be tested at a concentration of 2×10^6 cfu/mL. In a 96-well plate, 100 μL of 2× TSB-C, 50 μL each of the antibiotic dilutions, and the organism suspension are mixed and incubated at 37 °C for 18–24 h. The lowest concentration showing inhibition of growth was considered the MIC of the organism (13).

Ethidium Bromide Uptake Assay. WT *Fn*, *lpxD1*-null, and *lpxD2*-null were grown overnight in TSB-C broth at 25 °C and inoculated for a further 5 h in fresh medium. Cells were harvested and resuspended in PBS. The optical density at 600 nm was measured and adjusted to a final optical density reading of 0.1. Aliquots of the individual bacterial backgrounds (180 μL) were transferred to the 96-well plate. Twenty microliters of ethidium bromide (EtBr) (final concentration: 2.5 μM, 5 μM, 10 μM) was added to each of the individual wells. Fluorescence was read from the bottom of the wells using excitation and emission filter of 535 nm and 595 nm, respectively. Readings were taken at 2 min per time over a total of 30 min at 25 °C (13).

Organ Bacterial Burden. Mice were injected s.c. with *lpxD1*-null mutant and killed at indicated time points. Spleen and liver were harvested, homogenized, and serially diluted. The serial dilutions were then plated on TSB plates supplemented with 0.1% cysteine and plates were incubated at 37 °C for 24–72 h before determination of colony forming units (cfu).

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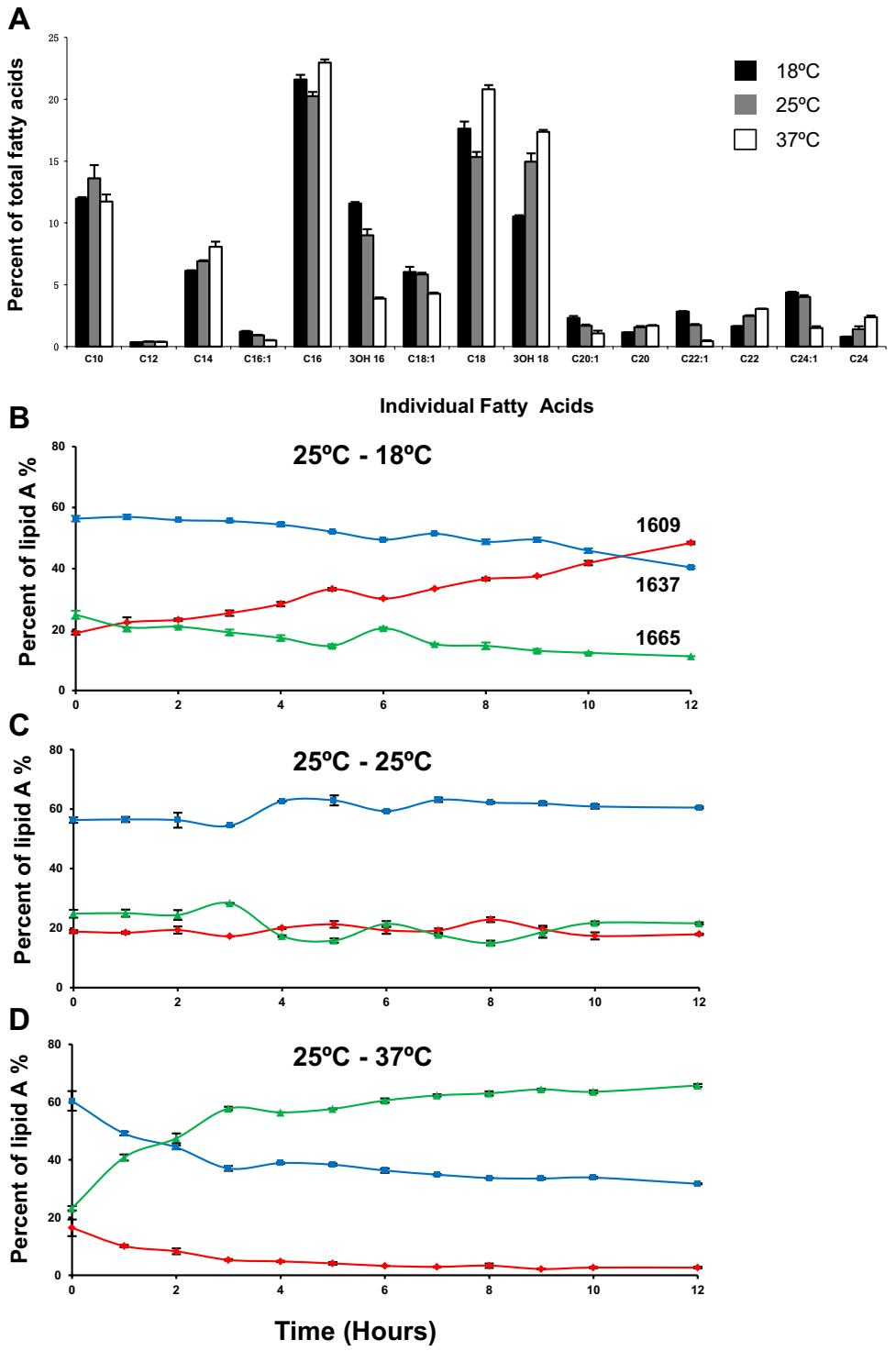
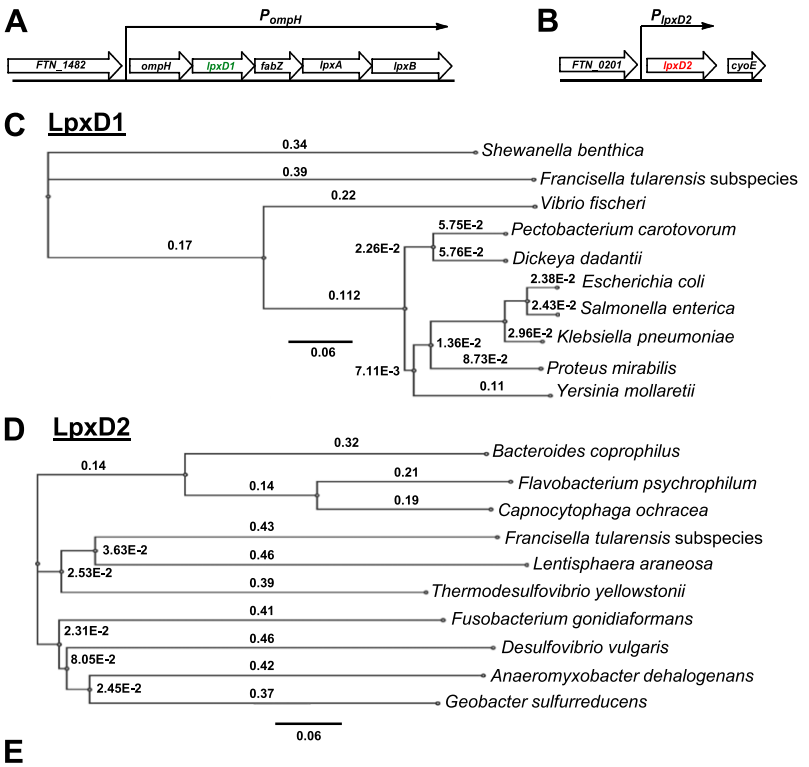


Fig. S1. (*A*) GC analysis of total acyl chains composition of *Fn*, *lpxD1-null*, and *lpxD2-null* mutant grown at 18 °C (black), 25 °C (gray), and 37 °C (white). (*B–D*) Overall membrane remodeling of *Fn* lipid A species after growth at different temperatures. Red diamond, lipid A ion at *m/z* 1609.1; blue circle, lipid A ion at *m/z* 1637.1; green triangle, lipid A ion at *m/z* 1665.2. (*B*) Percentage of individual lipid A species isolated at 25 °C then switched to 18 °C. (*C*) Percentage of individual lipid A species isolated at 25 °C. (*D*) Percentage of individual lipid A species isolated at 25 °C then switched to 37 °C. For all data points ($n = 3 \pm$ SE).



E

Gene name	Genome	Locus tag	Protein length (AA)	% identity (AA)
LpxD1	*Francisella novicida* U112	FTN_1480	337	100
LpxD1	*Francisella tularensis holarctica* OSU18	FTH_0539	337	99.4
LpxD1	*Francisella tularensis holarctica* FTA	FTA_0570	337	99.4
LpxD1	*Francisella tularensis tularensis* FSC 198	FTF1571c	337	99.4
LpxD1	*Francisella tularensis* WY96-3418	FTW_0355	337	99.1
LpxD1	*Francisella tularensis tularensis* SchuS4	FTT1571c	337	99.4
LpxD2	*Francisella novicida* U112	FTN_0200	347	100
LpxD2	*Francisella tularensis holarctica* OSU18	FTH_0191	347	98.9
LpxD2	*Francisella tularensis holarctica* FTA	FTA_0211	347	99.1
LpxD2	*Francisella tularensis tularensis* FSC 198	FTF0286c	347	97.4
LpxD2	*Francisella tularensis tularensis* WY96-3418	FTW_1800	347	98.3
LpxD2	*Francisella tularensis tularensis* SchuS4	FTT0286c	347	97.4

Fig. S2. Genetic organization and phylogenetic tree analysis of LpxD1 and LpxD2. Genetic organization of (*A*) *lpxD1* and (*B*) *lpxD2* locus in *Fn* genome, showing *lpxD1* lies within one operon and *lpxD2* is on the other side of the genome and is driven by its own promoter. (*C*) Phylogenetic tree analysis of LpxD1. (*D*) Phylogenetic tree analysis of LpxD2. (*E*) Conservation of LpxD in *Francisella* subspecies.

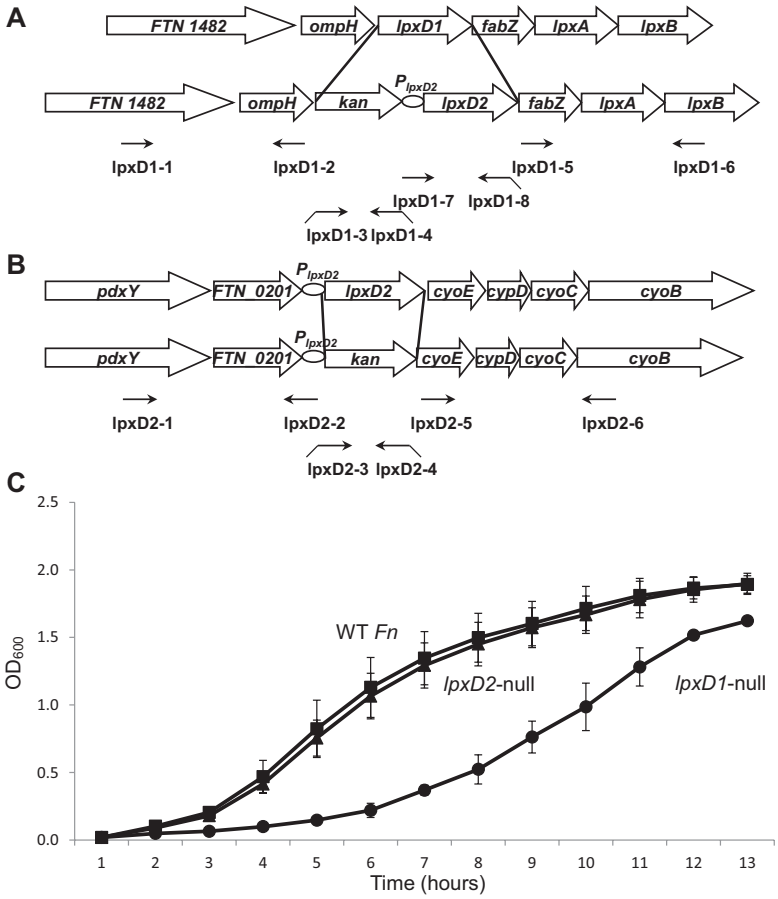
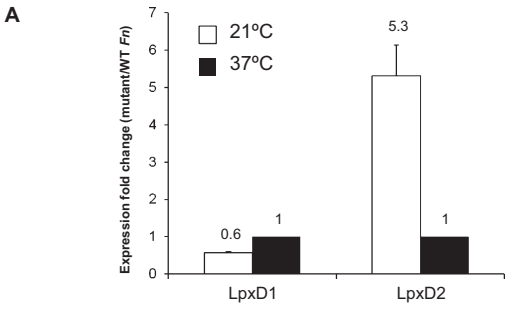


Fig. S3. Generation of (*A*) *lpxD1*-null and (*B*) *lpxD2*-null mutant. (*C*) Growth curves of *lpxD1*-null, *lpxD2*-null, and WT *Fn* at 37 °C.

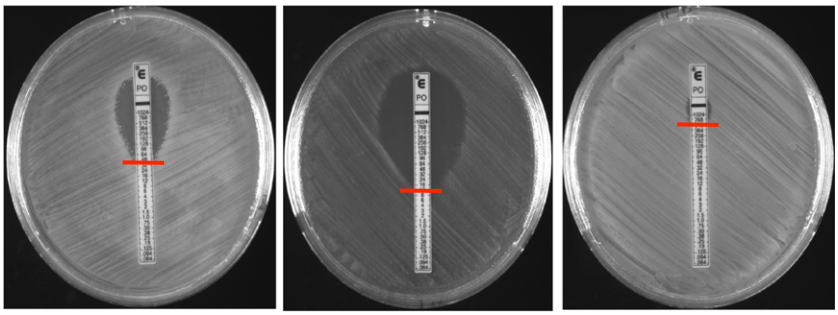


B

Hydroxyacyl-ACP Selectivity of Purified *Fn* LpxD					
	specific activity (nmol/min/mg)			selectivity ratio	
Protein	***R, S*-3-OH-C_{14}-ACP**	***R, S*-3-OH-C_{16}-ACP**	***R, S*-3-OH-C_{18}-ACP**	**C16/C14**	**C18/C14**
LpxD1	4.9 ± 3	7.5 ± 1	15.4 ± 1	1.51	3.13
LpxD2	9.6 ± 1	10.7 ± 1	5.7 ± 1	1.12	0.59

Fig. S4. (*A*) Quantitative PCR of *lpxD1* and *lpxD2* at 21 °C and 37 °C. (*B*) Hydroxyacyl ACP selectivity of purified *Fn* LpxD enzymes.

A



WT *Fn* *lpxD1*-null *lpxD2*-null

B

Minimum Inhibitory Concentration (ug/ml)

	Wild-type	*lpxD1*-null	*lpxD2*-null
Chloramphenicol	0.8 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
Carbenicillin	125.0 ± 10.0	62.5 ± 10.0	31.3 ± 10.0
Ciprofloxacin	0.3 ± 0.1	0.02 ± 0.01	0.3 ± 0.1
Erythromycin	3.1 ± 0.1	0.08 ± 0.05	0.08 ± 0.05
Rifampin	0.6 ± 0.1	0.02 ± 0.01	0.6 ± 0.1
Vancomycin	100 ± 10.0	6.3 ± 1	100 ± 10.0

C

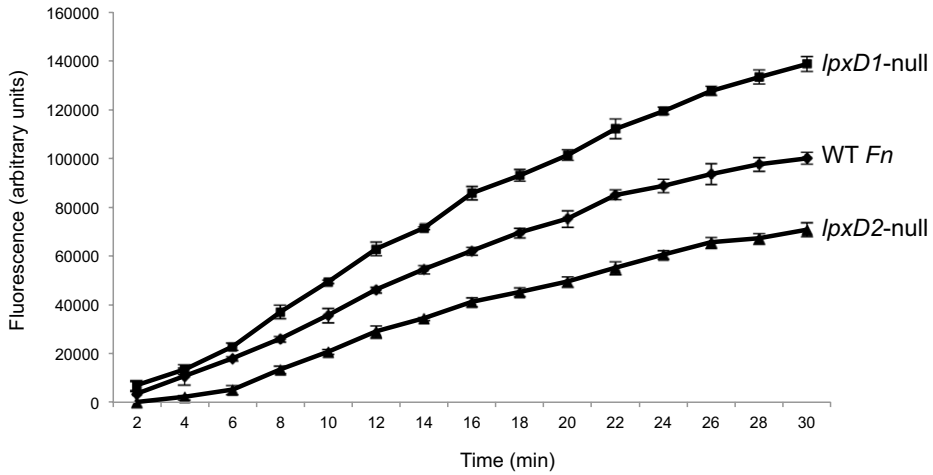


Fig. S5. Antibiotic resistance test of *lpxD1*-null and *lpxD2*-null *Fn* mutants. (*A*) Polymyxin B hypersensitivity of *lpxD1*-null, *lpxD2*-null, and WT *Fn*. (*B*) Antibiotic minimum inhibitory concentration (MIC) of *lpxD1*-null, *lpxD2*-null, and WT *Fn*. (*C*) Ethidium bromide uptake assay for *lpxD1*-null, *lpxD2*-null, and WT *Fn*.

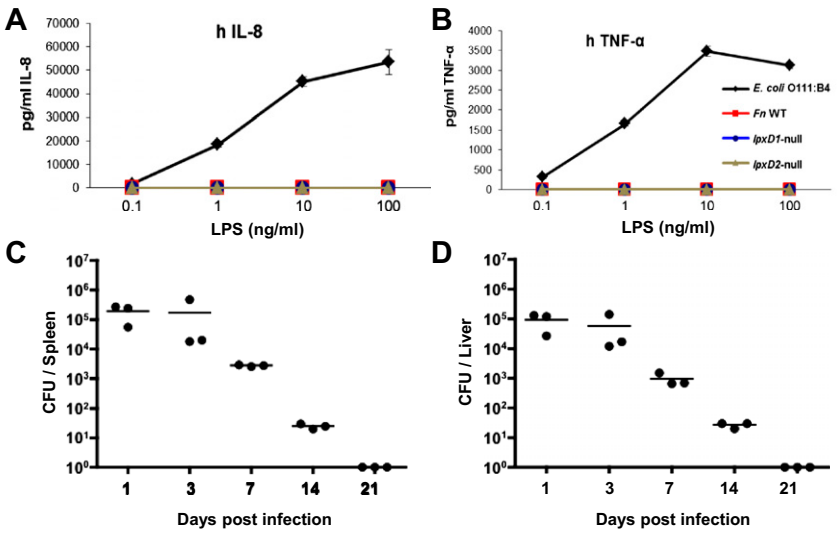


Fig. S6. (*A*) IL-8 and (*B*) TNF-α production by human macrophages THP-1 cell after stimulation with LPS isolated from *lpxD1*-null, *lpxD2*-null, WT *Fn* LPS, or purified *E. coli* O111:B4 LPS (positive control). Bacterial burden in C57BL/6 (*C*) liver and (*D*) spleen after s.c. infection with *lpxD1*-null *Fn* mutant (5.7×10^6 cfu).

Table S1. Fragment ion intensities from selected linear ion trap MS^n spectra

Table S1

Table S2. Microarray data

Table S2

Table S3. Bacterial strains and plasmids used in this work

Table S3