LPS remodeling is an evolved survival strategy for bacteria

Yanyan Li[a,b], Daniel A. Powell[b], Scott A. Shaffer[c], David A. Rasko[d], Mark R. Pelletier[b], John D. Leszyk[c], Alison J. Scott[b], Ali Masoudi[e], David R. Goodlett[f], Xiaoyuan Wang[a], Christian R. H. Raetz[e,1], and Robert K. Ernst[b,2]

[a]State Key Laboratory of Food Science and Technology, The Key Laboratory of Carbohydrate Chemistry and Biotechnology and Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214211, China; [b]Department of Microbial Pathogenesis, University of Maryland, Baltimore, MD 21201; [c]Departments of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605; [d]Institute for Genome Sciences, Department of Microbiology and Immunology, University of Maryland, Baltimore, MD 21201; [e]Department of Biochemistry, Duke University, Durham, NC 27710; and [f]Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195

Edited by Emil C. Gotschlich, The Rockefeller University, New York, NY, and approved April 18, 2012 (received for review February 24, 2012)

Maintenance of membrane function is essential and regulated at the genomic, transcriptional, and translational levels. Bacterial pathogens have a variety of mechanisms to adapt their membrane in response to transmission between environment, vector, and human host. Using a well-characterized model of lipid A diversification (*Francisella*), we demonstrate temperature-regulated membrane remodeling directed by multiple alleles of the lipid A-modifying *N*-acyltransferase enzyme, LpxD. Structural analysis of the lipid A at environmental and host temperatures revealed that the LpxD1 enzyme added a 3-OH C18 acyl group at 37 °C (host), whereas the LpxD2 enzyme added a 3-OH C16 acyl group at 18 °C (environment). Mutational analysis of either of the individual *Francisella lpxD* genes altered outer membrane (OM) permeability, antimicrobial peptide, and antibiotic susceptibility, whereas only the *lpxD1*-null mutant was attenuated in mice and subsequently exhibited protection against a lethal WT challenge. Additionally, growth-temperature analysis revealed transcriptional control of the *lpxD* genes and post-translational control of the LpxD1 and LpxD2 enzymatic activities. These results suggest a direct mechanism for LPS/lipid A-level modifications resulting in alterations of membrane fluidity, as well as integrity and may represent a general paradigm for bacterial membrane adaptation and virulence-state adaptation.

Microbial pathogens have evolved adaptive mechanisms to environmental changes (i.e., temperature, osmolality, pH, and concentrations of specific ions) encountered upon host entry. These mechanisms include modifications of the bacterial cell membrane to enhance the ability to colonize, localize to specific tissues, and avoid the host immune defenses. Bacteria coordinately regulate these survival mechanisms under environmental conditions in which they confer a selective or pathogenic advantage (1, 2). For many bacterial pathogens, an important environmental stimulus is a shift in temperature from vector or environment to the human host. Temperature change has been demonstrated to induce bacterial membrane remodeling and is required for maintenance of optimal membrane architecture (3–5).

Lipopolysaccharide (LPS, endotoxin) is the major component of the outer leaflet of the outer membrane (OM) of Gram-negative bacteria (5–7). LPS has three structural regions: O-antigen, core, and lipid A. Lipid A is the biologically active component of LPS recognized by the innate immune system (8). Gram-negative bacteria with both environmental and mammalian reservoirs can synthesize modified forms of lipid A in response to environmental stimuli and temperature change (1, 6, 7). Lipid A modifications can alter the bacterium's outer membrane integrity, susceptibility to antimicrobial peptides, immune stimulation, and pathogenesis. *Yersinia pestis*, the causative agent of the plague maintained among rodent populations and transmitted by infected fleas, synthesizes an alternate lipid A in different growth temperatures, a hexaacylated lipid A during growth at flea temperature (21 °C), and a tetraacylated lipid A upon growth at mammalian temperature (37 °C) (9–12). Each lipid A structural variant has been demonstrated to play a distinct role in the stage-specific pathogenesis of the organism. Specifically, increased acylation of lipid A at lower temperatures may protect the bacteria from conditions in the flea digestive tract or external environment, whereas decreased acylation allows the bacteria to evade detection by the host innate immune system. Induction of the innate immune system by modifying the tetraacylated lipid A structure by overexpression of the late acyltransferase, LpxL in *Y. pestis* results in a complete loss of virulence (13). In contrast, it has been demonstrated that *Francisella* expresses a unique tetraacylated LPS (14, 15) that fails to activate Toll-like receptor 4 (TLR4) at all growth temperatures due to the lack of the 1-position phosphate moiety and the length and position of the acyl groups attached to the diglucosamine backbone, enabling this Gram-negative organism to evade host detection (16).

We and others have previously demonstrated for *Francisella* that temperature plays an important role in altering the composition of lipid A (15, 17, 18). Upon growth at low temperatures (25 °C or lower), a mannose residue was added to the non-reducing glucosamine on the lipid A backbone and extensive heterogeneity in the composition and position of the acyl chains (18) was observed. This suggests a role for an environmentally regulated remodeling pathway of LPS in bacterial pathogenesis.

Francisella tularensis (*Ft*) subspecies *tularensis*, *Francisella holarctica* subspecies *holarctica*, and *Francisella novicida* (*Fn*) (19) have been identified in a wide array of cold- and warm-blooded hosts (fresh water protozoans, arthropods, and mammals), indicating remarkable adaptability, which is often associated with genomic and transcriptomic diversity (19–21). Transmission to a mammalian host results in a change in growth conditions that can induce transcriptional, translational, and posttranslational alterations affecting manifestations of bacterial pathogenesis (22). These alterations are required to maintain proper membrane function and impact the phospholipid and lipid A composition of the membrane. To determine the role of temperature in modulating *Francisella* membrane remodeling, we used the extensive characterization of environmentally regulated heterogeneity of *Fn* lipid A as a basis for studies presented herein.

In this study, we demonstrate temperature-regulated membrane remodeling directed by the lipid A-modifying *N*-acyltransferase enzymes LpxD1/2. Expression of the LpxD1 enzyme

Author contributions: Y.L. and R.K.E. designed research; Y.L., D.A.P., S.A.S., D.A.R., M.R.P., and J.D.L. performed research; Y.L. contributed new reagents/analytic tools; Y.L., S.A.S., D.A.R., M.R.P., A.J.S., A.M., D.R.G., C.R.H.R., and R.K.E. analyzed data; and Y.L., D.A.R., A.J.S., D.R.G., X.W., and R.K.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Deceased August 16, 2011.

[2]To whom correspondence should be addressed. E-mail: rkernst@umaryland.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202908109/-/DCSupplemental.

results in the incorporation of longer acyl chains (18 carbons in length) at the 2 and 2′ positions of lipid A, whereas expression of the LpxD2 enzymes adds shorter chain fatty acids (16 carbons in length) at the same positions. These modifications to lipid A manifest in the bacteria as compromised membrane integrity and permeability ultimately affecting pathogenicity and conferring protection against a lethal *Fn* challenge.

Results

Environmentally Regulated Remodeling of Lipid A. To observe the alteration of lipid A structures during transmission from an environmental source to a mammalian host, the lipid A synthesized by *Fn* after growth at the representative temperatures, 18 °C (environment), 25 °C (insect), and 37 °C (mammalian) was analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) in the negative ion mode (23). Three lipid A patterns were identified by MALDI-TOF MS, associated with growth at 18 °C, 25 °C, and 37 °C (Fig. 1). As previously demonstrated, lipid A extracted after growth at 37 °C showed four major $[M-H]^-$ ions at *m/z* 1609, 1637, 1665 (dominant), and 1827 (Fig. 1*A*). The dominant ion at *m/z* 1665 corresponds to a tetraacylated lipid A with two amide-linked 3-

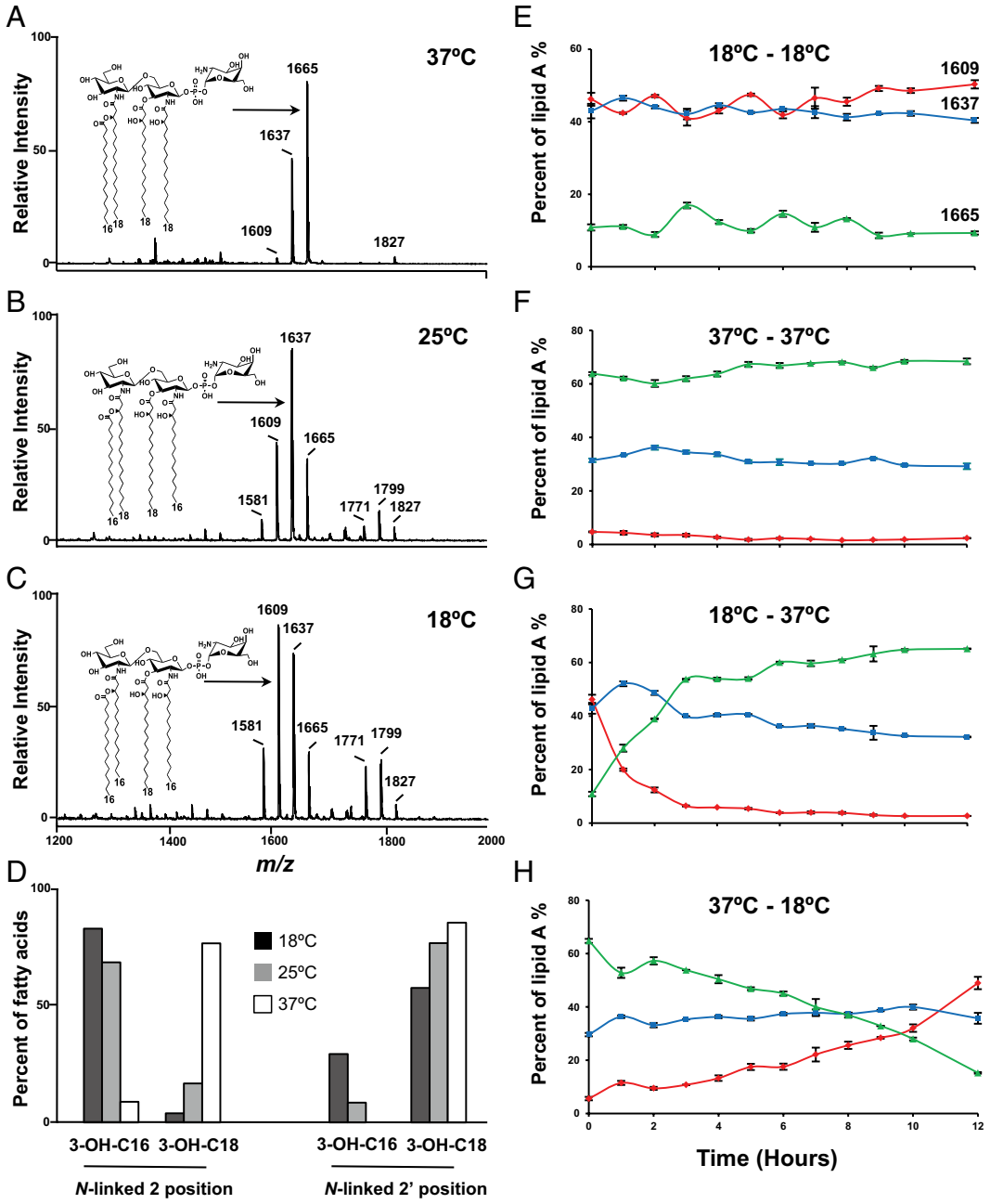

Fig. 1. Characterization of temperature-regulated structural modifications of *Fn* lipid A by negative ion MALDI-TOF MS. (*A*) MS from *Fn* grown at 37 °C; dominant lipid A at *m/z* 1665. (*B*) MS from *Fn* grown at 25 °C; dominant lipid A at *m/z* 1637. (*C*) MS from *Fn* grown at 18 °C; dominant lipid A at *m/z* 1609. (*D*) Acyl group frequency (*f*) for 2 and 2′ positions of *Fn* grown at 18 °C (black), 25 °C (gray), and 37 °C (white) ($n = 2$). (*E*) Percentage of lipid A species isolated at 18 °C. Red diamond, *m/z* 1609; blue circle, *m/z* 1637; green triangle, *m/z* 1665. (*F*) Percentage of lipid A species isolated at 37 °C. (*G*) Percentage of lipid A species isolated at 18 °C then switched to 37 °C. (*H*) Percentage of lipid A species isolated at 37 °C then switched to 18 °C. For all data points (*E–H*) ($n = 3 \pm$ SE).

OH-C18 (17, 24, 25). For lipid A extracted after growth at 25 °C, the major peaks were identified at *m/z* 1609, 1637 (dominant), 1665, 1771, 1799, and 1827 (Fig. 1*B*), and at 18 °C, were *m/z* 1581, 1609 (dominant), 1637, 1665, 1771, 1799, and 1827 (Fig. 1*C*). After growth at reduced temperatures, the size of the individual dominant peaks (Fig. 1 *A–C*) decreases by 28 Da (two methylene groups C_2H_4) indicating a decrease in the overall length of acyl chains (*m/z* 1665→1637→1609) on the glucosamine backbone. Additionally, increases of 162 Da indicated the addition of a mannose moiety at the 4′ position (15, 18).

Previously, the *m/z* 1665 ion was determined to be a single structure, whereas some of the other ion peaks represent complex heterogeneous mixtures of lipid A structures (18, 25). Estimates of the relative abundance of each structure present in an individual ion peak were derived from relative abundance and fragment ion ratios as previously calculated (18). Following quantitation of the frequency of acyl chains at 2 and 2′ positions (Fig. 1*D*), 3-OH-C18 was the dominant substituent at these positions following growth at 37 °C. In contrast, 3-OH-C16 was the dominant substituent after growth at 18 °C. A complete structural analysis for the individual ion peaks is presented in Table S1. Acyl chain alterations were confirmed by gas chromatography (GC) analysis (Fig. S1*A*) (26). These results clearly demonstrate that *Fn* modifies its lipid A structure in response to temperature adaptation by altering the length of the amide-linked acyl chains: 3-OH-C16 at environmental temperature and 3-OH-C18 at mammalian temperature.

Kinetics of Lipid A Remodeling. To investigate the kinetics of lipid A remodeling, *Fn* was initially grown at 18 °C (double time - 3.8 h) or 37 °C (double time - 1.5 h) and switched to an alternate temperature (18 °C to 37 °C, Fig. 1*G*) or (37 °C to 18 °C, Fig. 1*H*). Lipid A was extracted at 30-min intervals and analyzed by MALDI-TOF MS (23). The dominant lipid A peaks (*m/z* 1609, 1637, and 1665) were used to quantify the relative percentage of each structure. For *Fn* grown only at 18 °C, the major ions observed were *m/z* 1609 and 1637 (Fig. 1*E*), whereas at 37 °C, the major ion was *m/z* 1665 (Fig. 1*F*). Within 2.5 h of switching the temperature from 18 °C to 37 °C (double time - 1.7 h), the overall acyl chain content of lipid A was remodeled, and the dominant lipid A species was altered to contain the longer acyl chain containing molecule (Fig. 1*G*). Similarly, lipid A extracted after a switch from 37 °C to 18 °C contained the shorter acyl chain form of lipid A (Fig. 1*H*), however this conversion required 11 h (double time - 5.6 h). As expected, the intermediate temperature of 25 °C showed alterations with mixed levels of short and long acyl chains (Fig. S1 *B–D*). These results show that the bacteria are growing, synthesizing new lipid A molecules in response to the temperature shift, and are most likely engaging components of the lipid A biosynthetic pathway (Raetz pathway, Fig. 2*A*).

Duplication of LpxD, an *N*-Acyltransferase Lipid A Biosynthesis Enzyme. Bioinformatic analysis of *Ft* subspecies genomes identified two *N*-acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of *N*-linked acyl chains to lipid A at the 2 and 2′ positions, designated LpxD1 and LpxD2 (Fig. 2*A*). These enzymes catalyze the third step of lipid A biosynthesis (5) and are conserved among all Gram-negative bacteria. At least one functional LpxD is essential for bacterial viability (27–29). The *lpxD1*gene (*FTN_1480*) lies within an essential four-gene operon (*lpxD1/fabZ/lpxA/lpxB*, Fig. S2*A*). Interestingly, a second *lpxD* gene was identified, *lpxD2* (*FTN_0200*) as a single gene predicted to be regulated by its own promoter (Fig. S2*B*). *Fn* LpxD1 and LpxD2 share 34% amino acid sequence identity and a similar gene arrangement among respective *Francisella* subspecies (>98% amino acid sequence identity, Fig S2*E*), suggesting a conserved role in lipid A biosynthesis in this genus. Phylogenetic analyses indicated that *Fn* LpxD1 is similar to LpxD

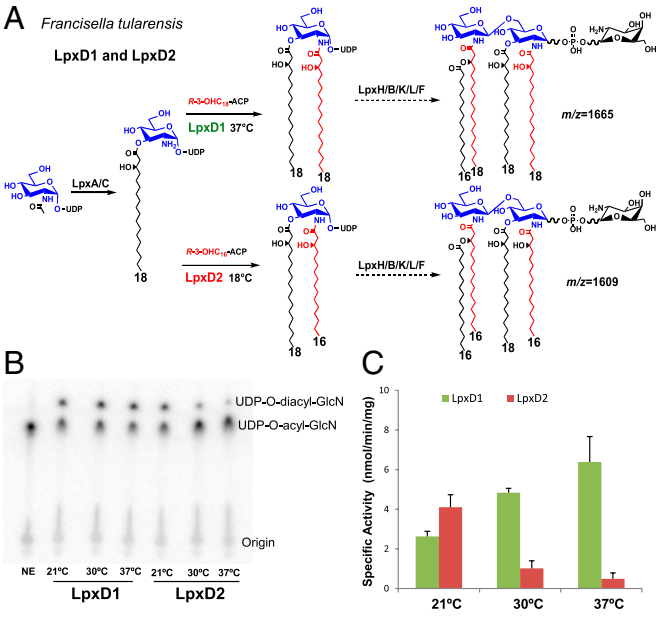

Fig. 2. Identification and temperature regulation of two *N*-acyltransferases LpxD1 and LpxD2 in *Fn*. (*A*) Lipid A biosynthesis pathway in *Fn*. (*B*) Conversion of [α-^{32}P] UDP-3-O-acyl-GlcN to [α-^{32}P] UDP-3-O-diacyl-GlcN by LpxD1/2 at 21 °C, 30 °C, and 37 °C. (*C*) Specific activity of LpxD1/2 at 21 °C, 30 °C, and 37 °C. Substrate conversion was quantified with ImageQuant software and used for the specific assay calculation. For all data points (n = 3 ± SE).

present in enteric species, although distinct and on its own deep branch (Fig. S2*C*), whereas the *Fn* LpxD2 is related to LpxD from anaerobic subspecies, such as *Flavobacteriae*, *Fusobacterium*, and *Desulfovibrio* (Fig. S2*D*). The unique gene arrangement and origins of LpxD in *Fn* may represent an adaptive response acquired through horizontal gene transfer and may be responsible for some of the observed heterogeneity in lipid A of *Fn*.

Acyl Chain Selectivity of *lpxD1*-Null and *lpxD2*-Null Mutant Strains of *Fn*. To determine the role of the individual LpxD enzymes in the synthesis of lipid A, *lpxD1*-null (Δ*lpxD1*) and *lpxD2*-null (Δ*lpxD2*) *Fn* mutant strains were generated by allelic replacement (30) (Fig. S3*A*). To maintain functional architecture of the *lpxD1/fabZ/lpxA/lpxB* operon, the entire *lpxD1* gene was replaced by the *lpxD2* gene fused to a kanamycin resistance cassette, resulting in the duplication of *lpxD2*; construction of this mutant as a single knockout strain unexpectedly resulted in a lethal phenotype at 37 °C, although lethality was not confirmed at lower growth temperatures. For the Δ*lpxD2* mutant strain, the *lpxD2* gene was replaced by the kanamycin resistance cassette (Fig. S3*B*). Lipid A isolated from either the Δ*lpxD1* or Δ*lpxD2* mutants showed markedly different MS profiles after growth at different temperatures (18 °C, 25 °C, and 37 °C) compared with the wild-type (WT) *Fn*. Negative ion MS analysis of lipid A isolated from the Δ*lpxD1* mutant after growth at all temperatures showed a dominant peak at *m/z* 1609 (Fig. 3 *A–C*), corresponding to a lipid A molecule with the shorter acyl chains (3-OH-C16) attached at the 2 and 2′ positions. In contrast, the dominant ion observed for the Δ*lpxD2* mutant was *m/z* 1665 (Fig. 3 *E–G*), corresponding to a lipid A molecule with longer acyl chains (3-OH-C18). Complementation *in trans* with a WT copy of the respective *lpxD* gene (pMP831-*lpxD1* or -*lpxD2*) resulted in the synthesis of the previously observed WT lipid A structure in these null mutant strains. These results strongly suggest that the LpxD acyltransferase enzymes are responsible for the temperature-regulated alterations in the environmental or mammalian lipid A phenotype with LpxD1 for the addition of longer acyl chains and LpxD2 for

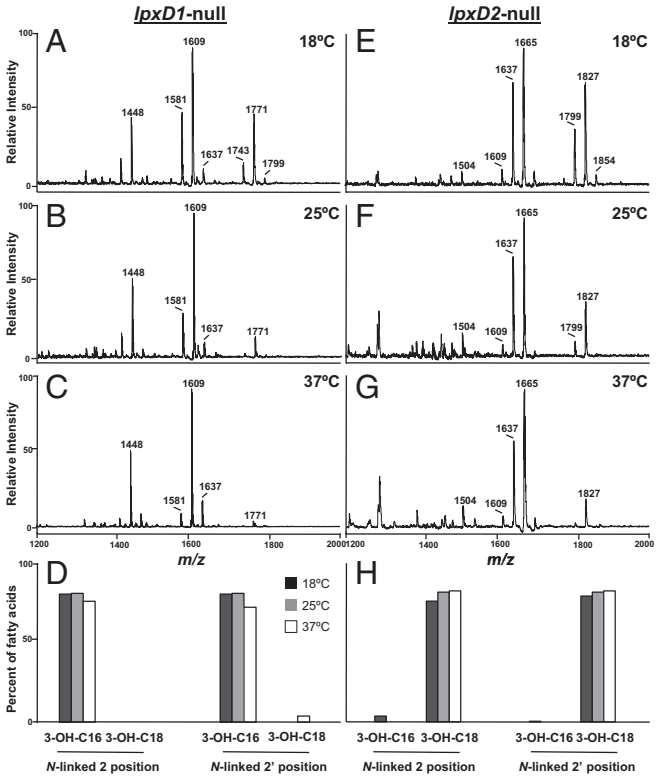

Fig. 3. Characterization of *Fn* lipid A from *lpxD1*-null and *lpxD2*-null mutants by negative ion MALDI-TOF MS. MS of lipid A from *Fn lpxD1*-null mutant grown at 18 °C (*A*), 25 °C (*B*), and 37 °C (*C*), respectively. Dominant lipid A peaks were locked at *m/z* 1609. (*D*) Acyl group frequency (r) for 2 and 2′ positions of *lpxD1*-null *Fn* grown at 18 °C (black), 25 °C (gray), and 37 °C (white). MS of lipid A from *Fn lpxD2*-null mutant grown at 18 °C (*E*), 25 °C (*F*), and 37 °C (*G*), respectively. Dominant lipid A was locked at *m/z* 1665. (*H*) Acyl group frequency (r) for 2 and 2′ position of *lpxD2*-null *Fn* grown at 18 °C (black), 25 °C (gray), and 37 °C (white). Data shown are representative of two independent analyses (*D* and *H*).

shorter acyl chains. The distribution (Fig. 3 *D* and *H*) and selectivity and relative amounts of the individual acyl chains were confirmed by MS analysis (Table S1).

Transcriptional Regulation of LpxD1 and LpxD2. The ability of *Ft* to cause disease in humans depends on its capacity to sense temperature change as it transmits from the environment to a warm-blooded host (21). Genome-wide expression profiles of *Fn* using custom microarray were determined after growth at environmental vs. mammalian temperatures to identify genes, including *lpxD1* and *lpxD2* involved in overall adaptation to temperature alterations. In total, ~17.8% of the genes were differentially regulated, 194 genes at environmental temperature and 106 genes at mammalian temperature (Table S2, LPS biosynthesis genes are highlighted). The *lpxD1* gene was specifically up-regulated at mammalian temperature (2.5-fold, Table S2), whereas *lpxD2* was up-regulated at environmental temperature (3.2-fold, Table S2). The results were confirmed by quantitative PCR (Fig. S4*A*). This demonstrates that the *lpxD* genes are transcriptionally regulated by temperature. In addition to *lpxD1/2*, *lpxB* (1.4-fold), *lpxC* (3.2-fold), *lpxF* (2.1-fold), and *flmF2* (2.9-fold) were up-regulated at environmental temperature (Table S2). These findings suggest a role in adaptation of the membrane for mannose-containing lipid A.

Enzymes Encoded by LpxD1 and LpxD2 Are Temperature-Dependent. To determine whether acyl chain length was regulated at the enzymatic level, LpxD1 and LpxD2 proteins were purified and activities were assayed (27, 28). LpxD1 enzymatic activity was 2.1-fold greater when assayed at 37 °C (5.1 nmol·min^{-1}·mg^{-1}) versus 21 °C (2.4 nmol·min^{-1}·mg^{-1}) (Fig. 2*B*). In contrast, LpxD2 enzymatic activity was 17.5-fold higher when assayed at 21 °C (3.5 nmol·min^{-1}·mg^{-1}) versus 37 °C (0.2 nmol·min^{-1}·mg^{-1}) as demonstrated by the increased addition of the short acyl chain, 3-OH-C16 at 21 °C (Fig. 2*C*). Additionally, analysis of the LpxD1/2 enzyme substrate specificity by the individual hydroxyacyl-acyl carrier proteins (ACP) showed similar temperature-dependent selectivity (Fig. S4*B*). This analysis combined with the previous mutant and transcriptional analyses demonstrate that the individual LpxD's are regulated by a temperature shift at both the transcriptional and enzymatic levels. These findings also demonstrate that multiple mechanism of regulation (transcriptional and enzyme activity) are used to rapidly adapt to an environmental stimuli for the maintenance of bacterial membrane fluidity and integrity, suggesting the importance for bacteria to finely control the composition and abundance of these membrane constituents.

Altered Antibiotic Susceptibility Patterns, Membrane Permeability, but Not Innate Immune Responses of *lpxD1*-Null and *lpxD2*-Null Mutants. Alteration in the composition of lipid A modulates recognition by host cationic antimicrobial peptides (CAMPs), susceptibility to antibiotics, and recognition by the host innate immune system (2). CAMPs target the bacterial membrane through electrostatic interactions. To study membrane susceptibility on the basis of LpxD alterations, we used polymyxin B (a cationic cyclic peptide with a acyl chain tail), which results in permeabilization of the membrane upon binding. Both Δ*lpxD1* and Δ*lpxD2* mutants were found to have altered susceptibility to this CAMP. The minimum inhibitory concentration (MIC) for the Δ*lpxD1* mutant was ~2.5-fold (14 μg/mL) less than that of the WT *Fn* (38 μg/mL) (Fig. S5*A*), whereas the Δ*lpxD2* mutant was ~13.5-fold (512 μg/mL) more resistant. Additionally, the Δ*lpxD1* mutant was more susceptible to antibiotics with diverse mechanisms of action such as chloramphenicol, carbenicillin, ciprofloxacin, erythromycin, rifampin, and vancomycin, whereas the Δ*lpxD2* mutant was only susceptible to carbenicillin and erythromycin (Fig. S5*B*). The most probable explanation for the differences in susceptibility is simply the diminished integrity of the LPS layer in the outer leaflet of the outer membrane, leading to higher permeability more readily allowing the diffusion of antibiotics (31, 32).

To examine this alteration in the outer membrane permeability (2), the MIC and uptake of ethidium bromide (EtBr) were measured. The EtBr MIC for growth of WT *Fn* was 3.9 μg/mL, whereas the Δ*lpxD1* and Δ*lpxD2* mutants were 0.5 μg/mL and 15.6 μg/mL, respectively. Compared with WT *Fn*, the Δ*lpxD1* mutant showed higher permeability to EtBr, whereas the Δ*lpxD2* mutant showed lower permeability (Fig. S5*C*).

Finally, LPS isolated from the Δ*lpxD1* and Δ*lpxD2* mutants was not recognized by components of the host innate immune system, similar to WT *Fn* LPS (14), suggesting that lipid A molecules with long chain acyl chains have altered binding properties to the LPS receptor, TLR4 complex (Fig S6 *A* and *B*). Therefore, even small changes to the outer membrane composition, such as shortening/lengthening specific acyl chain components, play an important role in modulating antibiotic susceptibility, membrane remodeling, and interactions with the host innate immune system.

***lpxD1*-Null Mutant Is Avirulent and Protective.** To investigate whether a primary infection with the *Fn lpxD* mutant strains displayed altered virulence, a murine model of infection was used (15). Groups of C57BL/6 mice were infected s.c. with ~500 cfu of WT *Fn* (~50 × LD_{100}), Δ*lpxD1* mutant, and Δ*lpxD2* mutant. The Δ*lpxD1* mutant was attenuated in mice, as all infected mice survived infection and showed no signs of disease (Fig. 4*A*). In

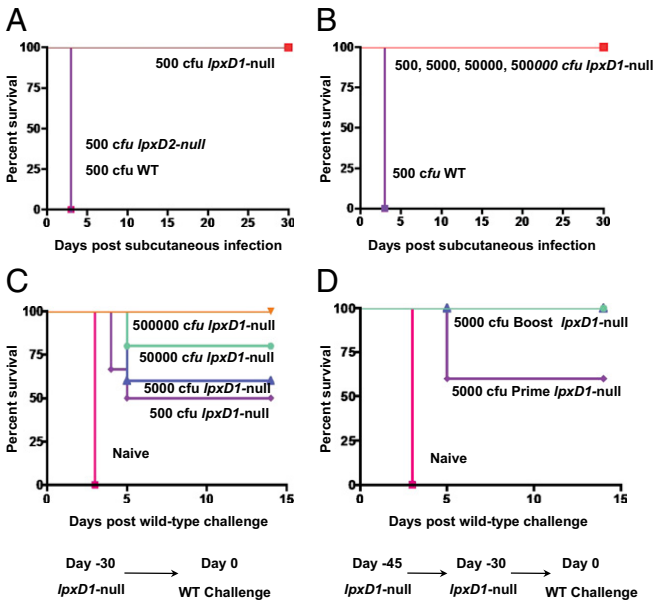

Fig. 4. Virulence assays of *lpxD1*-null and *lpxD2*-null *Fn* mutants in C57BL/6 mice. (*A*) Mice were inoculated with *lpxD1*-null, *lpxD2*-null, and WT *Fn* by the s.c. route. (*B*) Mice were inoculated with higher *lpxD1*-null mutant doses. (*C*) Mice were inoculated by s.c. injection on day −30 and subsequently challenged with WT *Fn* on day 0. (*D*) Mice were inoculated by s.c. injection on day −45 and day −14 and subsequently challenged with WT *Fn* on day 0. Data are representative of two independent experiments.

contrast, mice infected with the WT *Fn* or Δ*lpxD2* mutant succumbed by day 3 postinfection (p.i.) (Fig. 4*A*). Additionally, mice challenged s.c. with higher doses of Δ*lpxD1* mutant (~5,000; 50,000; and 500,000 × LD_{100}) (Fig. 4*B*) showed no signs of illness and uniformly survived the infection, showing the severe attenuation of this mutant. To confirm the role of LpxD1 enzyme in virulence, mice infected with the complemented strain, *lpxD1*-null/pMP831-*lpxD1* succumbed by day 3 p.i. The avirulent phenotype of the Δ*lpxD1* mutant was not due to attenuated growth in vitro, as this mutant strain achieved similar growth rates as the WT *Fn* by stationary phase of growth (Fig. S3*B*). To determine the kinetics of bacterial replication and dissemination, C57BL/6 mice were infected s.c. with WT *Fn* (50 × LD_{100}) or Δ*lpxD1* mutant (~500,000 × LD_{100}). Bacterial burdens in spleens and livers were determined. WT *Fn* was able to efficiently replicate in vivo (15); in contrast, there was no increase in the number of Δ*lpxD1 Fn* mutant bacteria in these organs at the early phase of infection. The overall number of bacteria steadily declined, becoming undetectable in all organs tested by day 21 p.i., indicating that a chronic or carrier state was not established (Fig. S6 *C* and *D*).

Because the Δ*lpxD1* mutant was attenuated in the murine model via s.c. route of infection, we investigated whether an Δ*lpxD1* mutant infection could protect mice from WT *Fn* challenge. Mice were inoculated s.c. with a single, escalating dose of the Δ*lpxD1* mutant on day −30 (500; 5,000; 50,000; and 500,000 cfu). These mice were then challenged with a lethal dose of WT *Fn* (500 cfu, ~50 × LD_{100}) and survival was recorded. Naïve controls all succumbed by day 3 postchallenge (p.c.), whereas mice previously infected with Δ*lpxD1* mutant showed a gradient of protection from the WT *Fn* challenge (Fig. 4*C*). Induction of protection was dependent on the primary infection dose of the Δ*lpxD1* mutant. Next, protection was determined using a prime-boost strategy to evaluate the potential for vaccine development using the Δ*lpxD1* mutant strain. Mice were initially primed s.c. on day −45 with a dose of Δ*lpxD1* mutant (~5,000 cfu), which partially protected the mice, followed by a booster inoculation on day −14 (~5,000 cfu) (Fig. 4*D*). Mice were then challenged with ~500 cfu of WT *Fn* on day 0 and survival was recorded. All mice that received a prime and boost inoculation of Δ*lpxD1* mutant survived WT challenge, whereas a single prime dose of the Δ*lpxD1* mutant on day −30 showed 60% mouse survival. All naïve controls died by 3 d p.i. (Fig. 4*D*). Therefore, the prime/boost immunization strategy with Δ*lpxD1* mutant elicits a protective immune response and suggests that this strain may be a candidate for further vaccine development.

Discussion

The lipid A component of LPS comprises the outer leaflet of the outer membrane in Gram-negative bacteria. The essential enzymes of the lipid A biosynthesis pathway are well conserved. In *Escherichia coli*, nine enzymes are required for biosynthesis of the major hexaacylated lipid A molecule (5). In *Francisella*, the lipid A biosynthesis pathway contains a duplication of the LpxD enzyme, designated LpxD1 and LpxD2. As *Francisella* synthesizes lipid A with an altered distribution of acyl chains in response to growth at environmental versus host temperatures, we were interested in elucidating the role of LpxD duplication, required for adding *N*-linked acyl chains (18, 25). In this paper, we demonstrate that the two acyltransferases, LpxD1/2, direct lipid A remodeling at the genomic, transcriptional, and translational levels. LpxD2 adds shorter acyl chains to lipid A, allowing adaptability in cold-blooded hosts, whereas LpxD1 adds longer acyl chains upon transmission to mammalian host temperatures. Interestingly, both *lpxD* genes are transcriptionally regulated and the enzyme activities of the LpxD proteins are temperature regulated, allowing for exquisite control over this process and suggesting that the process is critical for survival.

The OM of Gram-negative bacteria serves as a selective permeability barrier, to keep toxic compounds out of the cell while allowing vital nutrients and antibiotics to enter (2). To maintain proper membrane fluidity at low growth temperature, an enteric bacterial enzyme, LpxP incorporates a monounsaturated fatty acid (C16:1) at the 2′ acyl-oxo-acyl position. Mutation of LpxP resulted in altered OM composition and hypersusceptibility to antibiotics, suggesting altered membrane fluidity and permeability (33). Similarly, the Δ*lpxD1* mutant strain was found to be more susceptible to antibiotics and showed increased influx rates of ethidium dye, suggesting alteration of both the permeability and fluidity of the OM. In contrast to enteric bacteria, *Francisella* altered the chain length of the acyl groups located directly on the lipid A backbone. When restricted from remodeling its membrane, *Francisella* was avirulent in a mouse infection model. The acyl chain length modification of lipid A may alter folding, distribution, and interaction with outer membrane proteins which will be an area of future research.

The importance of the innate immune response in control of *Francisella* infection has been previously demonstrated (14). Lipid A modification enzymes, such as LpxF (30) and FlmK (15) have been demonstrated to play important roles in virulence but not in recognition by components of the host innate immune system, similar to WT *Fn* LPS (15).The *lpxF* and *flmK* deletion mutants displayed increased sensitivity to components of the host innate immune system and attenuation in murine infection models. To evaluate the role of LpxD1 and LpxD2 in pathogenesis, we tested deletion mutants in mice using a s.c. route of infection. We demonstrate that LpxD is required for full virulence as the Δ*lpxD1* mutant was attenuated in mice and protected mice against a lethal WT challenge. Interestingly, the Δ*lpxD1* mutant, which is permanently phase locked in the bacterial membrane of the environmental state, resulted in a direct LPS/lipid A orchestrated virulence defect. Taken together, these results highlight that even small changes to the outer membrane lipid A composition, such as shortening/lengthening of specific acyl chain components, play an

important role in modulating antibiotic susceptibility, membrane remodeling, and interactions with the host innate immune system.

These observations suggest a unique adaptive and regulatory mechanisms used by *Francisella* to modify membrane composition and modulate pathogenesis in response to a shift in temperature. As many Gram-negative bacterial pathogens survive in multiple environments before infecting a warm-blooded mammalian host, study of environmentally regulated alteration of the membrane architecture will allow elucidation of specific molecular details of the pathogenic mechanisms engaged upon transmission from the environment to the host.

Materials and Methods

Methods for LPS purification and lipid A isolation, mass spectrometry and gas chromatography procedures, mutant construction, RNA isolation and quantitative PCR, LpxD1 and LpxD2 expression and purification, cationic antimicrobial peptide sensitivity, antibiotic MIC test, ethidium bromide uptake assay, and organ bacterial burden are described in *SI Materials and Methods*.

Bacterial Strains and Growth Conditions. *F. novicida* U112 (*Fn*) obtained from Francis Nano (University of Victoria, Victoria, British Columbia, Canada) (34) was grown in TSB-C broth [3% tryptic soy broth and 0.1% cysteine (wt.vol)]. Antibiotics were added when required at the following concentrations: kanamycin (20 μg/mL) and hygromycin (100 μg/mL). For a complete list of strains and plasmids, see Table S3.

Mass Spectrometry Procedures. Lipid A structure was confirmed by negative ion MALDI analysis (CMBT matrix:CMBT, 5-chloro-2-mercaptobenzothiazole) on a Bruker Autoflex Speed. Estimates for the relative abundance of each structure were derived from MS relative abundance ratios and fragment ion ratios using comparable dissociation pathways as previously described (7).

LpxD Enzymatic Assay. The LpxD assay was carried out as previously reported (28). *R,S*-3-hydroxymyristoyl-ACP, *R,S*-3-hydroxypalmitoyl-ACP and [α-^{32}P]-UDP-3-*O*-(*R*-3-hydroxymyristoyl)-α-D-glucosamine were prepared as previously described (27, 28).

Virulence and Protection Studies. S.c. infections of mice C57BL/6 mice (The Jackson Laboratory) (n = 5) with WT, *lpxD1*-null, and *lpxD2*-null *Fn* mutant were carried out as previously described (15). For protection studies, mice were immunized with *lpxD1*-null mutant on day −45 and day −14 or on day −45 only, and challenged with a lethal dose of WT *Fn* on day 0 after the second immunization. Survival of the mice was recorded. All mice were housed in a specific pathogen-free facility. All protocols were approved by the institutional animal care and use committee of the University of Maryland, Baltimore.

ACKNOWLEDGMENTS. This study was supported by National Institutes of Health Grant U54 AI057141 (Region X – NWRCE), the National Natural Science Foundation of China (31170069), National Key Basic Research Program of China (2012CB725202), and the China Scholarship Council.

1. Miller SI, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol* 3:36–46.
2. Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 67:593–656.
3. Cybulski LE, Martín M, Mansilla MC, Fernández A, de Mendoza D (2010) Membrane thickness cue for cold sensing in a bacterium. *Curr Biol* 20:1539–1544.
4. Vigh L, et al. (2007) Membrane regulation of the stress response from prokaryotic models to mammalian cells. *Ann N Y Acad Sci* 1113:40–51.
5. Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* 76:295–329.
6. Trent MS, Stead CM, Tran AX, Hankins JV (2006) Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* 12:205–223.
7. Wang X, Quinn PJ (2010) Lipopolysaccharide: Biosynthetic pathway and structure modification. *Prog Lipid Res* 49:97–107.
8. Kusumoto S, Fukase K, Shiba T (2010) Key structures of bacterial peptidoglycan and lipopolysaccharide triggering the innate immune system of higher animals: Chemical synthesis and functional studies. *Proc Jpn Acad, Ser B, Phys Biol Sci* 86:322–337.
9. Rebeil R, et al. (2006) Characterization of late acyltransferase genes of *Yersinia pestis* and their role in temperature-dependent lipid A variation. *J Bacteriol* 188:1381–1388.
10. Krasikova IN, Khotimchenko SV, Solov'eva TF, Ovodov YS (1995) Mutual influence of plasmid profile and growth temperature on the lipid composition of *Yersinia pseudotuberculosis* bacteria. *Biochim Biophys Acta* 1257:118–124.
11. Salamah AA, Ali MA (1995) Effect of temperature on the lipid and fatty acid composition of *Yersinia pseudotuberculosis*. *New Microbiol* 18:27–33.
12. Kawahara K, Tsukano H, Watanabe H, Lindner B, Matsuura M (2002) Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect Immun* 70:4092–4098.
13. Montminy SW, et al. (2006) Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. *Nat Immunol* 7:1066–1073.
14. Hajjar AM, et al. (2006) Lack of in vitro and in vivo recognition of *Francisella tularensis* subspecies lipopolysaccharide by Toll-like receptors. *Infect Immun* 74:6730–6738.
15. Kanistanon D, et al. (2008) A *Francisella* mutant in lipid A carbohydrate modification elicits protective immunity. *PLoS Pathog* 4:e24.
16. Munford RS (2008) Sensing gram-negative bacterial lipopolysaccharides: A human disease determinant? *Infect Immun* 76:454–465.
17. Phillips NJ, Schilling B, McLendon MK, Apicella MA, Gibson BW (2004) Novel modification of lipid A of *Francisella tularensis*. *Infect Immun* 72:5340–5348.
18. Shaffer SA, Harvey MD, Goodlett DR, Ernst RK (2007) Structural heterogeneity and environmentally regulated remodeling of *Francisella tularensis* subspecies *novicida* lipid A characterized by tandem mass spectrometry. *J Am Soc Mass Spectrom* 18: 1080–1092.
19. Oyston PC, Sjostedt A, Titball RW (2004) Tularaemia: Bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* 2:967–978.
20. Hazlett KR, Cirillo KA (2009) Environmental adaptation of *Francisella tularensis*. *Microbes Infect* 11:828–834.
21. Keim P, Johansson A, Wagner DM (2007) Molecular epidemiology, evolution, and ecology of *Francisella*. *Ann N Y Acad Sci* 1105:30–66.
22. Horzempa J, Carlson PE, Jr., O'Dee DM, Shanks RM, Nau GJ (2008) Global transcriptional response to mammalian temperature provides new insight into *Francisella tularensis* pathogenesis. *BMC Microbiol* 8:172.
23. Li Y, Wang X, Ernst RK (2011) A rapid one-step method for the characterization of membrane lipid remodeling in using matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry. *Rapid Commun Mass Spectrom* 25:2641–2648.
24. Wang X, et al. (2006) Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. *Biochemistry* 45:14427–14440.
25. Schilling B, McLendon MK, Phillips NJ, Apicella MA, Gibson BW (2007) Characterization of lipid A acylation patterns in *Francisella tularensis, Francisella novicida*, and *Francisella philomiragia* using multiple-stage mass spectrometry and matrix-assisted laser desorption/ionization on an intermediate vacuum source linear ion trap. *Anal Chem* 79:1034–1042.
26. Bainbridge BW, et al. (2008) Acyl chain specificity of the acyltransferases LpxA and LpxD and substrate availability contribute to lipid A fatty acid heterogeneity in *Porphyromonas gingivalis*. *J Bacteriol* 190:4549–4558.
27. Bartling CM, Raetz CR (2009) Crystal structure and acyl chain selectivity of *Escherichia coli* LpxD, the N-acyltransferase of lipid A biosynthesis. *Biochemistry* 48:8672–8683.
28. Bartling CM, Raetz CR (2008) Steady-state kinetics and mechanism of LpxD, the N-acyltransferase of lipid A biosynthesis. *Biochemistry* 47:5290–5302.
29. Buetow L, Smith TK, Dawson A, Fyffe S, Hunter WN (2007) Structure and reactivity of LpxD, the N-acyltransferase of lipid A biosynthesis. *Proc Natl Acad Sci USA* 104: 4321–4326.
30. Wang X, Ribeiro AA, Guan Z, Abraham SN, Raetz CR (2007) Attenuated virulence of a *Francisella* mutant lacking the lipid A 4′-phosphatase. *Proc Natl Acad Sci USA* 104: 4136–4141.
31. Murata T, Tseng W, Guina T, Miller SI, Nikaido H (2007) PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar *typhimurium*. *J Bacteriol* 189:7213–7222.
32. Vaara M, Nurminen M (1999) Outer membrane permeability barrier in *Escherichia coli* mutants that are defective in the late acyltransferases of lipid A biosynthesis. *Antimicrob Agents Chemother* 43:1459–1462.
33. Carty SM, Sreekumar KR, Raetz CR (1999) Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction At 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J Biol Chem* 274:9677–9685.
34. Gray CG, Cowley SC, Cheung KK, Nano FE (2002) The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. *FEMS Microbiol Lett* 215: 53–56.