Correction

MICROBIOLOGY

The authors note that the following statement should be added to the Acknowledgments: “This material is based upon work supported by, or in part by, the US Army Research Laboratory and the US Army Research Office under Contract/Grant Number W911NF-11-1-0274.”

www.pnas.org/cgi/doi/10.1073/pnas.1212463109
LPS remodeling is an evolved survival strategy for bacteria

Yanyan Li,¹,² Daniel A. Powell,³ Scott A. Shaffer,³ David A. Rasko,⁴ Mark R. Pelletier,⁴ John D. Leszyk,⁵ Alison J. Scott,⁶ Ali Masoudi,⁶ David R. Goodlett,⁶ Xiaoyuan Wang,⁶ Christian R. H. Raetz,⁵,⁶ and Robert K. Ernst*¹,²

¹State 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, Jiaonan University, WuXi 214221, China; ²Department of Microbial Pathogenesis, University of Maryland, Baltimore, MD 21201; ³Departments of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605; ⁴Institute for Genome Sciences, Department of Microbiology and Immunology, University of Maryland, Baltimore, MD 21201; ⁵Department of Biochemistry, Duke University, Durham, NC 27710; and ⁶Department 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. Versinia 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 hexacarlylated 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 mannosamine 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 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.


The authors declare no conflict of interest.

1Deceased August 16, 2011.
2To 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. 1A). 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 (ґ) 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 ± 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. 1B), and at 18 °C, were m/z 1581, 1609 (dominant), 1637, 1665, 1771, 1799, and 1827 (Fig. 1C).

After growth at reduced temperatures, the size of the individual dominant peaks (Fig. 1A–C) decreases by 28 Da (two methylene groups -CH₂) 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 mannos 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 quantification of the frequency of acyl chains at 2 and 2′ positions (Fig. 1D), 3-OH-C18 was the dominant substrate at these positions following growth at 37 °C. In contrast, 3-OH-C16 was the dominant substrate 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. S1A) (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. 1G) or (37 °C to 18 °C, Fig. 1H). 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. 1E), whereas at 37 °C, the major ion was m/z 1665 (Fig. 1F). 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. 1G). Similarly, lipid A was altered after a switch from 37 °C to 18 °C contained the shorter acyl chain form of lipid A (Fig. 1H), 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. 24).

**Duplication of LpxD, an N-Acyltransferase Lipid A Biosynthesis Enzyme.** Bioinformatic analysis of Fl subspecies genomes identified two N-acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzymes, on the basis of sequence homology, that were predicted to be responsible for the addition of acyltransferase enzym...
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 peptide with a acyl chain tail), which results in permeabilization of the membrane upon binding. Both ΔlpxD1 and ΔlpxD2 mutants were found to have altered susceptibility to this CAMP. The minimum inhibitory concentration (MIC) for the ΔlpxD1 mutant was ∼2.5-fold (14 µg/mL) more resistant than that of the WT F. novicida (38 µg/mL) (Fig. S5A), whereas the ΔlpxD2 mutant was ∼13.5-fold (512 µg/mL) more resistant. Additionally, the ΔlpxD1 mutant was more susceptible to antibiotics with diverse mechanisms of action such as chloramphenicol, carbenicillin, ciprofloxacin, erythromycin, rifampin, and vancomycin, whereas the ΔlpxD2 mutant was only susceptible to carbencillin and erythromycin (Fig. S5B). 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 F. novicida was 3.9 µg/mL, whereas the ΔlpxD1 and ΔlpxD2 mutants were 0.5 µg/mL and 15.6 µg/mL, respectively. Compared with WT F. novicida, the ΔlpxD1 mutant showed higher permeability to EtBr, whereas the ΔlpxD2 mutant showed lower permeability (Fig. S5C).

Finally, LPS isolated from the ΔlpxD1 and ΔlpxD2 mutants was not recognized by components of the host innate immune system, similar to WT F. novicida 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 F. novicida 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 F. novicida (∼50 × 10^10), ΔlpxD1 mutant, and ΔlpxD2 mutant. The ΔlpxD1 mutant was attenuated in mice, as all infected mice survived infection and showed no signs of disease (Fig. 4A). In
contrast, mice infected with the WT Fn or ΔlpxD2 mutant succumbed by day 3 postinfection (p.i.) (Fig. 4A). Additionally, mice challenged s.c. with higher doses of ΔlpxD1 mutant (∼5,000; 50,000; and 500,000 × LD₀₀₀) (Fig. 4B) 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 ΔlpxD1 mutant was not due to attenuated growth in vitro, as this mutant strain achieved similar growth rates as the 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.

Because the ΔlpxD1 mutant was attenuated in the murine model via s.c. route of infection, we investigated whether an ΔlpxD1 mutant infection could protect mice from WT Fn challenge. Mice were inoculated s.c. with the single, escalating dose of the ΔlpxD1 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 × LD₀₀₀) and survival was recorded. Naïve controls all succumbed by day 3 postchallenge (p.c.), whereas mice previously infected with ΔlpxD1 mutant showed a gradient of protection from the WT Fn challenge (Fig. 4C). Induction of protection was dependent on the primary infection dose of the ΔlpxD1 mutant. Next, protection was determined using a prime-boost strategy to evaluate the potential for vaccine development using the ΔlpxD1 mutant strain. Mice were initially primed s.c. on day −45 with a dose of ΔlpxD1 mutant (∼5,000 cfu), which partially protected the mice, followed by a booster inoculation on day −14 (∼5,000 cfu) (Fig. 4D). 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 ΔlpxD1 mutant survived WT challenge, whereas a single prime dose of the ΔlpxD1 mutant on day −30 showed 60% mouse survival. All naïve controls died by 3 d p.i. (Fig. 4D). Therefore, the prime/boost immunization strategy with ΔlpxD1 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 hexacylated 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-alkyl 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 ΔlpxD1 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 ΔlpxD1 mutant was attenuated in mice and protected mice against a lethal WT challenge. Interestingly, the ΔlpxD1 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 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 Naco (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.