Leptospira interrogans lpxD Homologue Is Required for Thermal Acclimatization and Virulence

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15

ABSTRACT

Leptospirosis is an emerging disease with an annual occurrence of over 1 million human cases worldwide. Pathogenic *Leptospira* are maintained in zoonotic cycles involving a diverse array of mammals with the capacity to survive outside the host in aquatic environments. Survival in the diverse environments encountered by *Leptospira* likely requires various adaptive mechanisms. Little is known about *Leptospira* outer membrane modification systems, which may contribute to the capacity of these bacteria to successfully inhabit and colonize diverse environments and animal hosts. *Leptospira* encode two genes annotated as UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferases (la0512 and la4326, *lpxD1* and *lpxD2*), that in other bacteria are involved in the early steps of lipid A biosynthesis, the membrane lipid anchor of lipopolysaccharide. Inactivation of only one of these genes, la0512/*lpxD1*, imparted sensitivity to host physiological temperature (37°C) and rendered the bacteria avirulent in an animal infection model. Polymyxin B sensitivity assays revealed compromised outer membrane integrity in the *lpxD1* mutant at host physiological temperature, but structural analysis of lipid A in this mutant revealed only minor changes in the lipid A moiety compared to that found in the wild type strain. In accord, an *in-trans* complementation restored phenotypes to a level comparable to the wild type strain. These results suggest that the gene annotated as *lpxD1* in *L. interrogans* plays an important role in temperature adaptation and virulence in the animal infection model.

INTRODUCTION

Leptospira are spirochete bacteria encompassing saprophytic and pathogenic species and are considered to be the most widespread zoonotic bacteria world-wide (1). *Leptospira* are the etiological agents of the disease leptospirosis which in severe manifestation leads to hemorrhage

in the lungs, meningitis, liver and/or kidney failure (1). Leptospirosis is an emerging disease and the worldwide annual occurrence is estimated to be over 1 million human cases with a 5-20% mortality rate (2, 3). *Leptospira* cannot breach the host epidermal lining and transmission requires direct contact of the bacteria with cuts or abrasions in the skin (4, 5). Rats and other rodent species serve as reservoir hosts for *Leptospira*, which colonize the urinary system of these animals (6). *Leptospira* are shed back into the environment through the urine of reservoir hosts and can persist in fresh water and soil until direct contact with an animal recommences an infection cycle (7, 8).

A prominent *Leptospira* feature is the ability to proliferate in significantly different environmental conditions. Other Gram-negative bacterial pathogens with this unique ability include species of the genera *Escherichia* (9), *Salmonella* (10), *Yersinia* (11), *Vibrio* (12) and *Pseudomonas* (13). The capacity of bacteria to adapt to disparate environments is likely imparted by numerous evolved strategies that likely include modification of outer membrane macromolecules (14-17). Outer membrane fluidity and permeability is partly modulated by hydrophobic acyl chains of lipid A regions of lipopolysaccharide (LPS) (18). Environmental fluctuations such as temperature alter outer membrane fluidity (19) and bacteria counter by altering acyl chain lengths, the number of acyl groups added to the lipid A moiety and/or the modification of acyl chain saturation, to maintain outer membrane integrity (20-22).

Measurements of the toxicity of *L. interrogans* LPS suggest that while the molecule is less toxic than *Escherichia coli* LPS (23), *L. interrogans* LPS is toxic to a variety of cells (24) and tissues encountered by the pathogen during the course of animal infection (25). *L. interrogans* LPS is also an immunodominant molecule (26, 27) and unique in that it is recognized by TLR2 and not TLR4 in human cells (LPS from the majority of other Gram

62 negative bacteria is recognized by TLR4) (28). Pathogenic *Leptospira* demonstrate more
63 abundant and longer LPS compared to the saprophyte *L. biflexa* (29) and mutations affecting the
64 native biosynthesis of LPS affect both virulence in hamsters (30) and colonization of target
65 organs in the mouse model (31).

66 Acylation of lipid A has been shown to be crucial for the fitness of bacteria outside and
67 within the host (15, 20, 21). The *L. interrogans* genome encodes homologs of the enzymes
68 required for lipid A biosynthesis and this biosynthetic process has been previously proposed in *L.*
69 *interrogans* (32). Structural analyses of *L. interrogans* serovars Pomona and
70 Icterohaemorrhagiae (strain Verdun) lipid A have been performed, revealing identical structures
71 composed of a 2,3-diamino-2,3-dideoxy-D-glucopyranose disaccharide with four amide linked
72 acyl groups composed of *R*-3-hydroxylaurate at positions 3 and 3′ and *R*-3-hydroxypalmitate at
73 positions 2 and 2′ (32). Two secondary unsaturated acyl chains are ester linked to the 2′ and 3′
74 hydroxy-acyl groups to produce hexa-acylated lipid A as the major species (32). Previous
75 structural analysis of *L. interrogans* lipid A suggested that the C2 and C2′ amine groups are
76 acylated with 16 carbon length hydroxy-acyl groups (32), which would suggest that the *L.*
77 *interrogans* LpxD enzyme is selective for 16 carbon 3-hydroxy-acyl chains. The *L.* interrogans
78 serovar Manilae examined in this report, have two genes (la0512 and la4326) that display
79 homology to *lpxD* in other Gram-negative bacteria. The present study aimed to characterize
80 pathogenic *L. interrogans* serovar Manilae *lpxD* homologs in the context of outer membrane
81 integrity conferring temperature adaptation and virulence in an animal infection model.

MATERIALS AND METHODS

Leptospira strains and culturing

Leptospira interrogans serovar Manilae strain L495, the la0512^{-} (*lpxD1*$^{-}$) and la4326^{-} (*lpxD2*$^{-}$) mutants, and la0512^{-} mutant complemented with la0512 (*lpxD1*$^{-/+}$) were maintained in EMJH growth medium at 30°C, with agitation.

Insertion mutagenesis and complementation.

Insertion inactivation in *L. interrogans* has been previously described (33). The insertion sites within la0512 (*lpxD1*$^{-}$) and la04326 (*lpxD2*$^{-}$) were identified by semi-random PCR followed by DNA sequencing. The insertion was further confirmed via PCR using primers flanking the insertion sites. For complementation, *lpxD1*$^{-}$ was PCR amplified using primers *lpxD1*F (5′-GGGAATTCCATATGAAAGCCAAAAATTTAGCG-3′) and *lpxD1*R (5′-CGGCTCGAGATCCAATTCAACCTG -3′), which incorporated restriction digestion sites for NdeI and XhoI, respectively (underlined bases). The *lpxD1* coding sequence was then digested with NdeI and XhoHI, purified, and inserted into the same restriction sites of pCRPromFlgB (34) to generate a transcriptional fusion between the gene and the *Borrelia burgdorferi flgB* promoter. DNA fragments containing the transcriptional fusions were released by KpnI and XhoI digestions and cloned into the corresponding sites of pAL614 (generous gift from Gerald Murray, Monash University). The *lpxD1* complementation construct was introduced by conjugation in Manilae *lpxD1*$^{-}$ strain as previously described (35) and complementation of the *lpxD1*$^{-}$ strain was confirmed by using primers that PCR amplified a region of the spectinomycin resistance cassette and primers *lpxD1*F and *lpxD1*R, using genomic DNA as template.

Growth rate measurements, susceptibility assays and morphology determination

To determine whether mutant, complemented and wild type *Leptospira* strains were affected in growth *in vitro*, bacteria were enumerated using a Petroff-Hausser Counting Chamber (Hausser Scientific Company, Horsham, Pennsylvania, USA) under dark field microscopy. A total of 2000 of each strain was used to inoculate 9 ml EMJH, in triplicate. Growth was monitored on a daily basis at 30˚C and 37˚C, by counting bacteria using a Petroff-Hausser counting chamber under dark field microscopy. For enumerating strains on a daily basis, equal volume aliquots of samples were pooled and counted in duplicate. Growth experiments were performed 4 times with similar trends, a representative result from a single experiment is displayed in Fig. 1.

Temperature sensitivity was measured by adding 200 µl of bacteria at a concentration of $5x10^6$ bacteria/ml in EMJH to 96 well culture plates, in triplicate. Plates were sealed to prevent evaporation and incubated at 23˚C, 30˚C or 37˚C for 72 h. To measure cell viability, bacterial cultures were transferred to a single 96 well culture plate and 20 µl of AlamarBlue (Life Technologies SAS, Saint Aubin, Île-de-France, France) was added to bacteria and plates incubated for 24 h at 30˚C. Temperature sensitivity experiments were performed twice with similar results and a representative result from a single experiment is displayed in Fig. 1. For polymyxin B (Sigma, Saint Louis, Missouri, USA) sensitivity assays experiments were performed as described for the temperature sensitivity assays with the following modifications. Polymyxin B was added to 96 well plates in the range of 20 µg/ml – 0.02 µg/ml, using 2 fold serial dilutions. Bacteria were added to a final volume of 200 µl and a final concentration of $5x10^5$ bacteria/ml. Plates were sealed to prevent evaporation and incubated at 30˚C or 37˚C for 24 h, and 20 µl of AlamarBlue was subsequently added per well and plates were sealed and incubated at 30˚C for 48 h to measure cell viability. For these experiments the starting bacterial

concentration was $5x10^5$ (as opposed to $5x10^6$ for temperature sensitivity assays above) to ensure *lpxD1*$^-$ viability at 37°C as the viability of this mutant rapidly declines with increasing bacterial concentration at 37°C. Polymyxin B experiments were performed two times with comparable results and a representative result from a single experiment is displayed in Fig. 2.

Morphological changes were measured by first growing bacterial strains at 23˚C, 30˚C or 37˚C to a density of $5x10^6$ bacteria/ml, in triplicate. Ten microliter aliquots of bacteria were viewed by dark field microscopy at 200x magnification and images captured using Olympus CellSens Dimensions version 1.7.1 (Olympus, Rungis, Île-de-France, France). Twenty bacteria were measured length-wise (using the CellSens Dimensions version 1.7.1 measuring tool) per replicate for a total of 60 bacteria per strain, from each temperature. Statistical analysis was performed using two-way ANOVA.

RNA extraction and RT-qPCR

Leptospira strains were cultured in triplicate at 30˚C in EMJH to a density of $1x10^8$ bacteria/ml and a total of 10^{10} of each strain from each replicate was used for RNA extraction and RT-qPCR, as previously described (35-38). The following modifications were implemented for RT-qPCR; the primers used to quantify respective genes included *lpxD1*f 5'-ATCCGAACGTTGTCATTGAA and *lpxD1*r 5'-GATCACCGTATTCGCATGAA to quantify *lpxD1*$^-$ transcripts and *lpxD2*$^-$f 5'-TCATCCTTCTGCAAAGTTGG and *lpxD2*$^-$r 5'-AACGCCGTCTTCCAAATAAG to quantify *lpxD2* transcripts. Statistical analysis was performed using both biological replicates (n=3) and RT-qPCR technical replicates (n=3) using unpaired Student's *t* –test, comparing strains individually.

LPS purification and immunoblot analyses

149 *Leptospira* strains were cultured at 37˚C in EMJH to a density of $1x10^7$ bacteria/ml and
150 harvested via centrifugation at 9,000 x*g* for 10 min to obtain a total of 10^{10} cells of each strain.
151 The LPS was purified as previously described (30) with the following modifications.
152 Immediately after harvesting, *Leptospira* pellets were resuspended in 1x PBS-0.1% SDS to a
153 concentration of 10^9 bacteria/ml and sonicated for 45 sec at 20 W. Proteinase K was added to a
154 final concentration of 30 µg/ml and samples were incubated at room temperature 24 h on a
155 RotoFlex Tube Rotator (Argos Technologies, Elgin, Illinois, USA). Samples were subsequently
156 mixed with 4x Laemmli protein sample buffer (Bio-Rad, Marnes-la-Coquette, Île-de-France,
157 France) and used for SDS-PAGE, Silver Staining and immunoblot analysis using an equivalent
158 volume of $1x10^7$ bacteria per lane. Immunoblots were performed using *Leptospira interrogans*
159 serovar Manilae strain L495 positive guinea pig sera which was obtained as previously described
160 (39), at a 1:100 dilution in 1x PBS/5%(w/v) skim milk/0.1% Tween-20 (PBSMT). Guinea pig
161 sera were detected with horse radish peroxidase-conjugated goat anti-guinea pig
162 immunoglobulins as previously described (39). These experiments were performed 3 times with
163 similar results and the result from a single experiment is displayed in Fig. S1.

164 **Isolation and MALDI-MS analysis of lipid A**

165 *Leptospira* strains were grown in EMJH media at 30˚C or 37˚C to a density of $3x10^7$
166 bacteria/ml and a total of $3x10^{10}$ of each strain from each temperature was used for lipid A
167 isolation. Lipid A isolation and mass spectrometry were performed as previously described (40)
168 with the following modifications. Cells were harvested by centrifugation, washed with PBS, and
169 stored at -20°C until lipid A extraction. Previously described modifications to the methods of
170 Caroff and Raetz were used to chemically isolate lipopolysaccharide, where isolated material
171 was treated by boiling mild-acid hydrolysis for 45 minutes to liberate lipid A from attached

polysaccharide (40-42). The method of Bligh and Dyer was used to extract lipid A after mild-acid hydrolysis (43). Isolated lipid A was dried under nitrogen and stored at -20°C until further use.

Lipid A extracts were further purified over a diethylaminoethyl (DEAE) column to improve the quality of spectra obtained by MALDI-MS. Briefly, dried lipid A was suspended in 2:3:1 v/v chlorofom:methanol:water (C:M:W) and applied to a 1.5 ml pre-equilibrated DEAE column. The column was washed with 20 column volumes of 2:3:1 C:M:W, and lipid A species were eluted stepwise with 5 column volumes of 2:3:1 C:M: ammonium acetate at 60 mM, 120 mM, 240 mM, or 480 mM ammonium acetate. An additional two-phase Bligh-Dyer extraction was performed on the eluate to remove ammonium acetate. Isolated lipid A was dried under nitrogen and stored in small conical glass vials at -20°C until MALDI-MS analysis. Lipid A was resuspended in 25 µl chloroform-methanol (4:1). An empirically determined amount of lipid A, varied per sample, was mixed with 0.5 µl of matrix (saturated 6-aza-2-thiothymine in 50% acetonitrile: saturated tribasic ammonium citrate (20:1, v/v)) and spotted on a 100 well MALDI plate. The AB SCIEX Voyager was used to collect MALDI-MS-TOF data in the negative reflectron mode. Consistent with previous reports in *L. interrogans*, m/z peaks corresponding to lipid A species were present in most of the 60 mM ammonium acetate fraction spectra (32). The proposed absence of the 4′-phosphate group and a methylated 1-phosphate contribute to the early elution of lipid A from *L. interrogans* (32). No peaks corresponding to lipid A were observed in the later 120, 240, 480 mM ammonium acetate fractions. Spectra obtained represent the average of >300 shots.

Gerbil infection and bacterial burden in target organs

194 An initial virulence experiment was performed in gerbils with Manilae wt strain and the
195 mutant strains *lpxD1*$^{-}$ and *lpxD2*$^{-}$. For these experiments, groups of 4 gerbils were challenged
196 intraperitoneally with each of the aforementioned strains using 10^4 bacteria per animal. Animals
197 were monitored for 20 days and euthanized, when possible, to minimize animal suffering. A
198 second infection experiment was performed as described above with the following modification:
199 complemented *lpxD1*$^{-}$ strain (*lpxD1*$^{-/+}$) was used in place of strain *lpxD2*$^{-}$ and infections were
200 performed at doses of 10^4 and 10^6 bacteria per animal, in groups of 4 gerbils per bacterial dose.

201 *Leptospira* burden in kidneys and liver were determined by qPCR, as previously
202 described (35, 44), with the following modifications. *Leptospira* strains Manilae wild type,
203 *lpxD1*$^{-}$ and *lpxD1*$^{-/+}$ were injected intraperitoneally into groups of 4 gerbils (10^4 bacteria per
204 animal). Five days post injection of bacteria, animals were euthanized and kidneys and liver from
205 each animal was harvested for culturing in EMJH and for qPCR. Calculations of bacterial burden
206 were performed to obtain the number of bacteria per 100 mg organ. F test to compare variance ($p < 0.0064$) was used to compare bacterial burden in target organs.

208 **Ethics statement**

209 The protocols for the animal experiments were prepared according to the guidelines of
210 the Animal Care and Use Committee of Institut Pasteur of Paris and the present study was
211 approved by this committee (N° CETEA 2013-0019).

212 **RESULTS**

213 ***lpxD1* inactivation reduces *L. interrogans* fitness at host physiological temperature**

LpxD enzymes have been shown to contribute to bacterial temperature adaptation via modification of acyl chain length of the lipid A region of lipopolysaccharide (15). To determine whether *lpxD* genes contained similar functionality in *Leptospira*, strains with *Himar1* transposon insertions in the genes la0512 and la4326 (*lpxD1*$^{-}$ and *lpxD2*$^{-}$, respectively) were obtained from a previously generated library of transposon insertion mutants (33, 45). I*n vitro* growth rates of mutant strains *lpxD1*$^{-}$ (la0512) and *lpxD2*$^{-}$ (la4326) were compared to parent Manilae L495 strain (wt) at 30˚C and 37˚C (Fig. 1A and B). These experiments showed comparable growth rates for both mutants and wt at 30˚C, however, at 37˚C reduced cell density was observed in mutant *lpxD1*$^{-}$ relative to wt and mutant *lpxD2*$^{-}$ (Fig. 1B). To further test mutant *lpxD1*$^{-}$ temperature susceptibility, mutant strains were cultured to a density of $5x10^{6}$/ml (approaching the approximate density at which point *lpxD1*$^{-}$ lost viability at 37˚C) in growth media and incubated at 23˚C, 30˚C and 37˚C for 72 h. Following the 72 h incubation, bacterial viability was measured via AlamarBlue viability assay which demonstrated that mutant *lpxD1*$^{-}$ was not viable at 37˚C (Fig. 1C).

To validate these observations, mutant *lpxD1*$^{-}$ was complemented with the native *lpxD1* gene under the control of a constitutive promoter (technical limitations prevented complementation with native *lpxD1* promoter). The resulting strain (*lpxD1*$^{-/+}$) displayed a surprisingly faster growth rate at 37˚C and reached a higher density at both 30˚C and 37˚C when compared to the mutant and wt strains (Fig. 1A and B). Quantitative RT-PCR (RT-qPCR) was used to measure relative *lpxD1* transcription in the respective strains, demonstrating a >2.5 fold increase in *lpxD1* transcription in *lpxD1*$^{-/+}$ when compared to wt (supplemental Fig. S1).

Increased outer membrane permeability in mutant *lpxD1*$^{-}$

236 Outer membrane integrity was assessed using polymyxin B assays conducted at 23˚C,
237 30˚C and 37˚C and the viability of the bacteria were determined using AlamarBlue assays (Fig.
238 2A). Interestingly, mutant *lpxD2*$^{-}$ displayed a 2 fold higher resistance to polymyxin B and this
239 resistance was independent of the tested temperatures (Fig. 2A). In contrast, mutant *lpxD1*$^{-}$
240 exhibited a 2 fold higher sensitivity to polymyxin B at 37˚C but not at 30˚C or 23˚C, when
241 compared to the other strains (Fig. 2A). The wt and *lpxD1*$^{-/+}$ strains displayed comparable
242 polymyxin B susceptibility (Fig. 2A).

243 Reduced outer membrane integrity can lead to osmotic stress resulting in reduced cell
244 size (46). To assess whether strains displayed morphological differences, the cell lengths of the
245 mutant, complement and wt strains were measured using dark field microscopy (supplemental
246 Fig. S2). These analyses revealed that at 37˚C the cell length of mutant *lpxD1*$^{-}$ was reduced by
247 16% (1.5 μm on average) when compared to the other strains (supplemental Fig. S2).

248 ***lpxD1* is required for leptospiral kidney and liver colonization and for fatal leptospirosis in**
249 **gerbils**

250 Immunoblot analysis using *Leptospira* positive sera and total LPS from mutant,
251 complement and wt strains grown at 37°C demonstrated reduced reactivity with LPS obtained
252 from mutant *lpxD1*$^{-}$ (Fig. 3), providing indirect evidence of *lpxD1* function in the host. To
253 ascertain whether *lpxD1* function was also required for survival of *Leptospira* in the host,
254 mutants *lpxD1*$^{-}$, *lpxD2*$^{-}$ and wt strain were used in virulence experiments in the gerbil infection
255 model (Fig. 4A). These experiment revealed that when animals were injected intraperitoneally
256 with 10^4 bacteria per gerbil, those infected with strain *lpxD1*$^{-}$ survived the 20 day infection
257 experiment (Fig. 4A), without visual signs of morbidity, whereas animals infected with *lpxD2*$^{-}$
258 and those with wt died by day 7. In separate experiments mutant *lpxD1*$^{-}$, complemented *lpxD1*$^{-/+}$

and wt strain were injected into the intraperitoneal cavity of gerbils at doses of 10^4 or 10^6 (Fig. 4B). Animals infected with strain *lpxD1*$^-$ at a dose of 10^4 and 10^6 per animal remained asymptomatic for the duration of the 20 day experiment whereas animals infected with wt or strain *lpxD1*$^{-/+}$ died at days 5 and 7 post infection, when injected with doses of 10^6 or 10^4 bacteria, respectively (Fig. 4B).

For analysis of bacterial burden in kidneys and liver, gerbils were injected intraperitoneally with 10^4 strain *lpxD1*$^-$, complement *lpxD1*$^{-/+}$ or wt strain and 5 days post infection animals were sacrificed to obtain kidneys and livers for quantitative real-time PCR (qPCR) and for *in vitro* culturing. Quantitative real-time PCR led to the detection of *lpxD1*$^-$ DNA in the kidneys and livers at an approximately ten fold lower quantity compared to wt (Fig. 4C). To distinguish between viable bacteria that had established colonization and those that reached the target organs but that died shortly after, organs were also used for *in vitro* culturing. The wt and *lpxD1*$^{-/+}$ strains were culture positive in all of the kidneys and livers tested but strain *lpxD1*$^-$ was negative for growth (data not shown).

Structural analyses of *Leptospira* lipid A

MALDI-MS analysis of lipid A species isolated from *L. interrogans* serovar Manilae produced data that is similar to previously published reports on serovars Pomona and Icterohaemorrhagiae (32). The previously proposed structure of *L. interrogans* lipid A is hexaacylated, contains a methylated 1-phosphate and lacks a 4′-phosphate group (33) (Fig. 5). Consistent with this structure, fractionation of *L. interrogans* serovar Manilae lipid A using anion exchange chromatography (DEAE) revealed early elution (60 mM ammonium acetate) of lipid A species consistent with a net decrease in anionic character due to the absence of a 4′ phosphate group and the presence of a methylated 1-phosphate group. Unmodified hexa-acylated

bis-phosphorylated lipid A typically elutes in the 240 mM ammonium acetate fraction during DEAE fractionation. Unique to our analysis of serovar Manilae is an overall reduction in the observed *m*/*z* relative to serovars Pomona and Icterohaemorrhagiae by *m*/*z* 2 (Fig. 6A; 37°C) or 4 (Fig. 7A; 30°C), consistent with the incorporation of fatty acids with 1 or 2 more degrees of unsaturation (Fig. 5). The location of the unsaturated bond cannot be determined by MALDI-MS.

In all spectra the predominant, putative lipid A species peak is flanked by less intense peaks at *m*/*z* -28 or +28 (Fig. 6 and Fig. 7), consistent with a lipid A species containing acyl chains that vary in length by 2 less or 2 more carbons, respectively. These peaks are also observable in MS data from previously characterized serovars Pomona and Icterohaemorrhagiae (32). In serovar Manilae an even shorter acyl-chain containing lipid A is present in *lpxD1*$^{-}$, which varies from the predominant peak by *m*/*z* -56, corresponding to a reduction in acyl chain length by 4 carbons (Fig. 6B and Fig. 7B). Complementation of the *lpxD1*$^{-}$ mutant with a wild type copy of *lpxD1* resulted in the disappearance of this peak at either temperature (30°C or 37°C), resulting in a spectrum similar to wild-type (Fig. 6C and Fig. 7C). Analysis of lipid A from *lpxD2*$^{-}$ resulted in a spectra very similar to that obtained for the wild-type at 37°C (Fig. 6D). However, the MALDI/MS spectra obtained from lipid A purified from mutant *lpxD2*$^{-}$ grown at 30°C consistently produced a spectra lacking the major spectral peaks (Fig. 7D) at *m*/*z* 1691, 1719 and 1747 observed in wild-type samples (Fig. 7A).

DISCUSSION

Phenotypic characterization of mutant and the complemented *lpxD1*$^{-/+}$ strains demonstrated that *lpxD1* is required for optimal bacterial growth and survival at temperatures corresponding to that found within the host (37°C). Furthermore, temperature sensitivity of

305 *lpxD1*¯ mutant at 37°C was at least partially a result of increased outer membrane permeability,
306 as demonstrated by polymyxin B susceptibility assays. We did not assess bacterial growth at
307 temperatures lower than 23°C, as *Leptospira* growth rate rapidly declines at lower temperatures
308 *in vitro*. We cannot therefore exclude the importance of *lpxD2* for bacterial viability at lower
309 temperatures. The use of two different LpxD enzymes for temperature adaptation has been
310 previously demonstrated in the Gram-negative bacterial species *Francisella* (15).

311 Consistent with the requirement for *lpxD1* functionality at 37°C, comparative
312 immunoreactivity of whole LPS molecules from wt, mutant and complemented strains suggested
313 that *lpxD1* is functional during the infection process in animals. Furthermore, virulence
314 experiments in animals indicated that *lpxD1* was essential for the ability of *Leptospira* to
315 colonize target organs and to cause fatal disease in gerbils. The lack of *lpxD1* expression and
316 functionality in the *lpxD1*¯ mutant likely rendered *Leptospira* inept in maintaining a stable outer
317 membrane for proper functioning of the bacteria at the elevated temperatures found within the
318 host. Reduced fitness within the host due to increased outer membrane permeability was also
319 likely further compounded by increased susceptibility to host antimicrobial peptides.

320 MALDI-MS analysis of lipid A species from all *Leptospira* strains used in the present
321 study demonstrated a somewhat heterogeneous mixture with respect to degrees of unsaturation
322 and overall acyl chain length. The predominant species observed corresponds to the proposed
323 structure in Fig. 5. Spectra from *lpxD1*¯ mutant strain displayed an additional lower *m*/*z* peak
324 suggestive of a lipid A moiety with shortened acyl chain lengths. The appearance of the smaller
325 acyl-chain containing lipid A species in the *lpxD1*¯ mutant might suggest that the protein product
326 of *lpxD2* (if indeed functional) has a more relaxed acyl chain substrate specificity than that of
327 *lpxD1*, allowing the incorporation of even smaller acyl chains into the final lipid A structure.

328 Comparison of the major lipid A species for *L. interrogans* serovar Manilae showed that at lower
329 temperatures the most predominant lipid A species at 30°C (m/z 1719.7) contained one more
330 degree of unsaturation relative to the predominant species at 37°C (m/z 1722.0). The
331 homeoviscous adaptation hypothesis for bacterial membranes predicts increased unsaturated
332 fatty acid content at low temperatures which promotes the necessary increase in fluidity, required
333 for optimal bacterial membrane functionality (22, 47). Direct biochemical characterization of
334 LpxD1 and LpxD2 will be required to determine the precise contribution of each enzyme to the
335 observed lipid A species in this study. It is also possible that changes in fatty-acid donor pools
336 (ratio of various acyl-ACPs) as a consequence of varying growth temperatures, or the activity of
337 secondary lipid A acyltransferases, LpxL or LpxM, might contribute to the observed changes in
338 lipid A acyl chain length/unsaturation.

339 The results of the present study are in agreement with previous theories on bacterial
340 mechanisms used for modulating outer membrane integrity for bacterial adaptation to new
341 environments. Contrary to other bacteria, such as *Yersinia* and *Neisseria*, the total number of *L.*
342 *interrogans* lipid A acyl chains appeared to remain constant under the various temperatures
343 tested. In the case of *L. interrogans*, the effect(s) of acyl chain length and level of acyl chain
344 saturation on thermal acclimatization and virulence in the animal infection model remains
345 unclear. These results allow us to extrapolate that *L. interrogans* likely utilize *lpxD1* function to
346 maintain outer membrane integrity when transmitted from niche inanimate environments, such as
347 soil and water, to the animal host.

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FIGURE LEGENDS

Fig. 1. Inactivation of *Leptospira lpxD1* results in temperature sensitive growth. *Leptospira* parent wt, mutant *lpxD1* (*lpxD1*$^{-}$) and *lpxD2* (*lpxD2*$^{-}$), and complemented *lpxD1* (*lpxD1*$^{-/+}$) strains were used to compare temperature sensitivity. (A) Strains were cultured in triplicate at 30°C and cell density measured for each strain by pooling replicates and counting cells using Petroff-Hauser counting chambers under dark field microscopy, daily. (B) Strains were cultured at 37°C and cell densities determined as for 30°C cultures. (C) Temperature sensitivity was also determined by exposing strains at a density of $5x10^6$/ml to the indicated temperatures for 72 h. Cell viability was subsequently measured using an alamarBlue assay where pink represents viable cells, blue non-viable cells and intermediate between pink and blue represents reduced viability

Fig. 2. Reduced outer membrane integrity in the *lpxD1*$^{-}$ mutant at 37°C. The relative outer membrane permeability of *Leptospira* parent wt, mutant *lpxD1* (*lpxD1*$^{-}$) and *lpxD2* (*lpxD2*$^{-}$), and complemented *lpxD1*$^{-}$ (*lpxD1*$^{-/+}$) strain was tested by measuring minimum inhibitory concentrations to the cationic compound polymyxin B. Bacteria were exposed to polymyxin B at the indicated concentration range (using two fold successive dilutions) for 72 h at the designated

507 temperatures. Cell viability was measured using the alamarBlue assay, viable bacteria are
508 represented in the pink wells and non-viable bacteria in blue wells.

509 **Fig. 3. Inactivation of *lpxD1* results in lowered immunoreactivity to LPS.** Crude LPS extracts
510 of bacteria grown at 37°C were applied to SDS-PAGE and used in immunoblot experiments with
511 sera from guinea pigs infected with wild type Manilae. (A) Silver stained SDS-PAGE of crude
512 LPS from the indicated strains. (B) Immunoblot displaying IgG reactivity of *Leptospira* positive
513 sera against crude LPS from indicated strains.

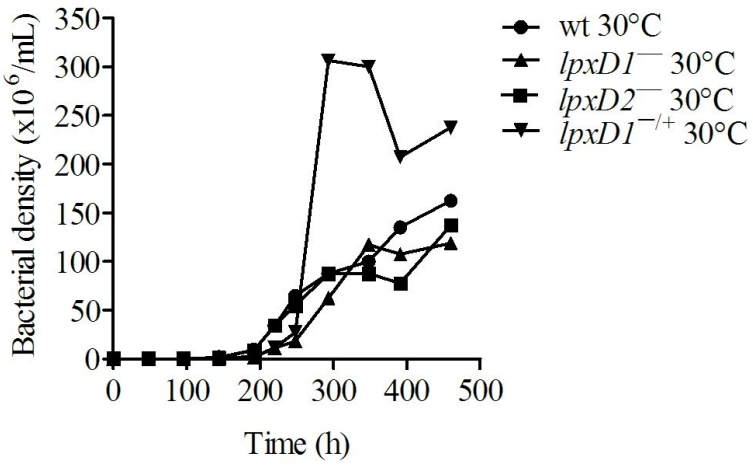
514 **Fig. 4. Mutant *lpxD1*$^-$ does not colonize target organs in the gerbil infection model.**
515 *Leptospira* parent wt, mutant *lpxD1* (*lpxD1*$^-$) and *lpxD2* (*lpxD2*$^-$), and complemented *lpxD1*
516 (*lpxD1*$^{-/+}$) strains were used to assess virulence in gerbils. (A) Groups of 4 gerbils were
517 inoculated with the indicated numbers of each strain, intraperitoneal and monitored for 20 days.
518 (B) Infection experiment were performed as described for panel A. (C) Groups of 4 gerbils were
519 inoculated intraperitoneal with 10^4 of each indicated strain per animal. Five days post
520 inoculation, animals were euthanized and livers and kidneys taken for qPCR and *in vitro*
521 culturing of bacteria in EMJH. [a]Culture positive from both liver and kidney, [b]Negative for
522 culture growth from liver and kidney.

523 **Fig. 5. Proposed chemical structure of the major lipid A species from *L. interrogans*.**
524 Chemical representations of the predominant species of lipid A in serovars Pomona and Verdun,
525 as proposed in a previous report (32), and serovar Manilae, the putative structure proposed in this
526 report. The exact mass, and the monoisotopic mass (m/z) for MS in the negative mode are
527 displayed.

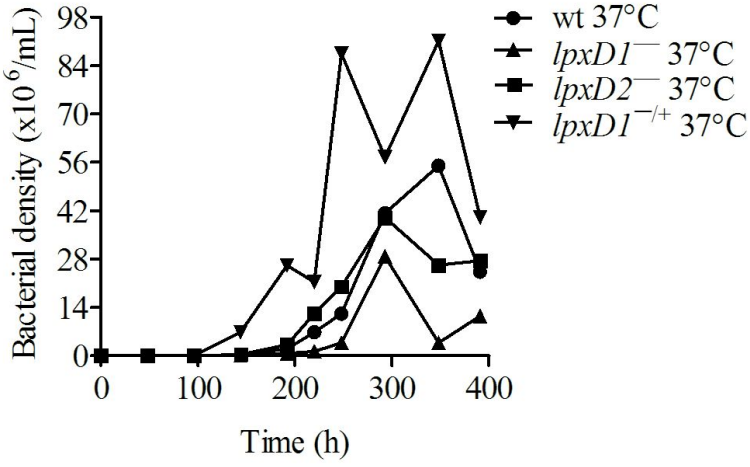
Fig. 6. MALDI-MS analysis of DEAE fractionated lipid A from *L. interrogans* serovar Manilae grown at 37°C. Lipid A isolated from each indicated strain was fractionated over a DEAE anion-exchange column. Mass spectra were obtained using the 60 mM ammonium acetate DEAE fraction for lipid A isolated from WT (A), *lpxD1*$^{-}$ (B), *lpxD1*$^{-/+}$ (C), and *lpxD2*$^{-}$ (D) strains of *L. interrogans* serovar Manilae. Each spectra is the average of >300 laser pulses. Based on relative % signal intensity, the most abundant m/z peak for each isotopic cluster is labeled.

Fig. 7. MALDI-MS analysis of DEAE fractionated lipid A from *L. interrogans* serovar Manilae grown at 30°C. Lipid A isolated from each indicated strain was fractionated over a DEAE anion-exchange column. Mass spectra were obtained using the 60 mM ammonium acetate DEAE fraction for lipid A isolated from WT (A), *lpxD1*$^{-}$ (B), *lpxD1*$^{-/+}$ (C), and *lpxD2*$^{-}$ (D) *L. interrogans* serovar Manilae. Each spectra is the average of >300 laser pulses. Based on relative % signal intensity, the most abundant m/z peak for each isotopic cluster is labeled.

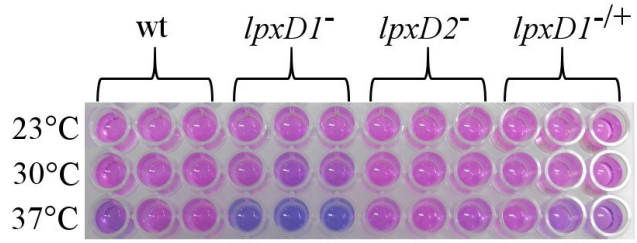
A

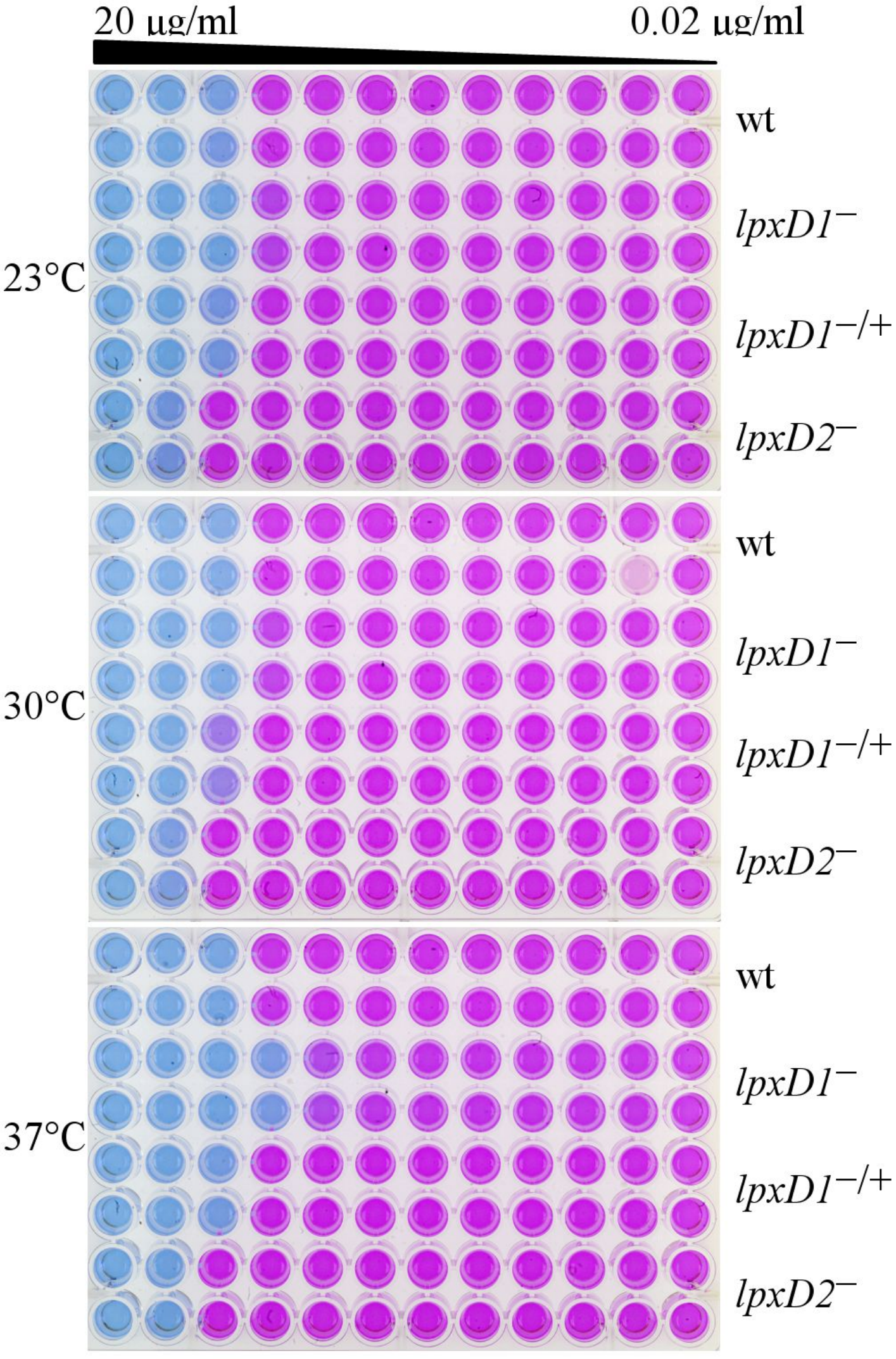

Bacterial density (x10^6/mL)
350
300
250
200
150
100
50
0
0
100
200
300
400
500
Time (h)
wt 30°C
lpxD1$^-$ 30°C
lpxD2$^-$ 30°C
lpxD1$^{-/+}$ 30°C

B

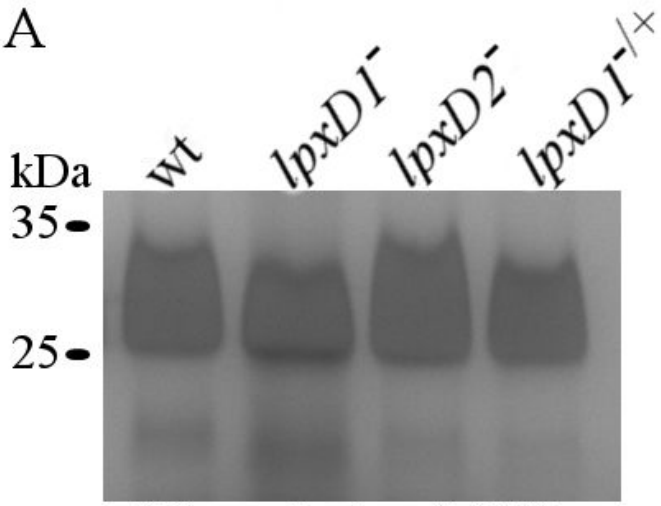

Bacterial density (x10^6/mL)
98
84
70
56
42
28
14
0
0
100
200
300
400
Time (h)
wt 37°C
lpxD1$^-$ 37°C
lpxD2$^-$ 37°C
lpxD1$^{-/+}$ 37°C

C


wt
lpxD1$^-$
lpxD2$^-$
lpxD1$^{-/+}$
23°C
30°C
37°C

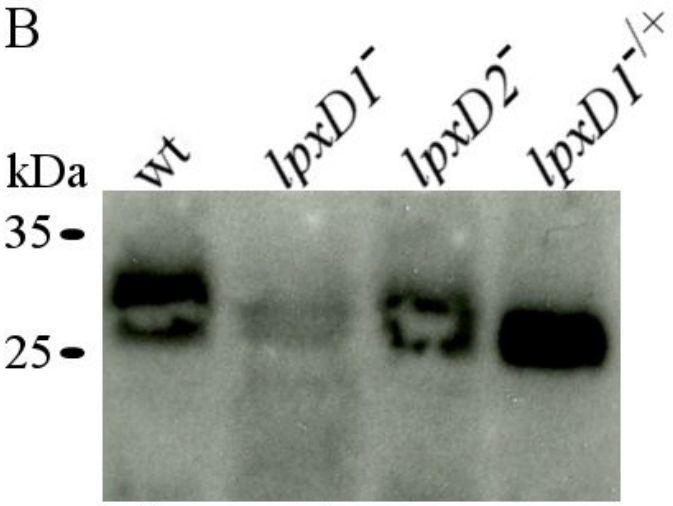

20 µg/ml
0.02 µg/ml
23°C
30°C
37°C
wt
lpxD1−
lpxD1−/+
lpxD2−
wt
lpxD1−
lpxD1−/+
lpxD2−
wt
lpxD1−
lpxD1−/+
lpxD2−

A

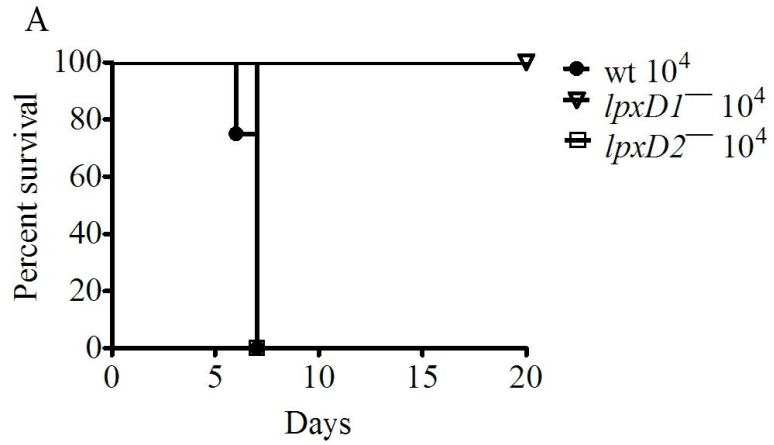


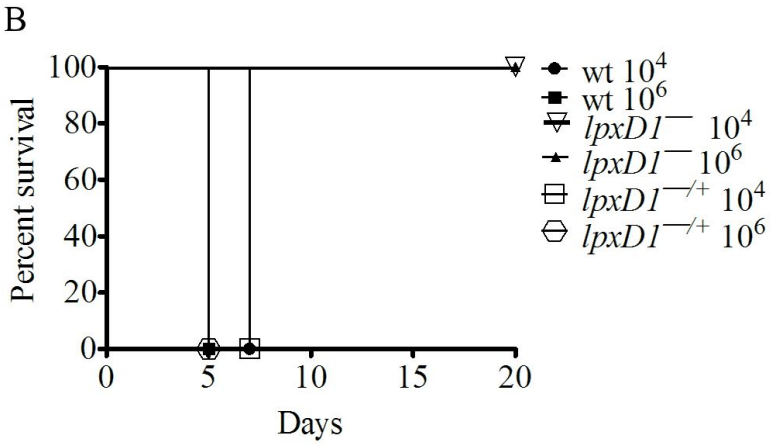
Silver Stained LPS

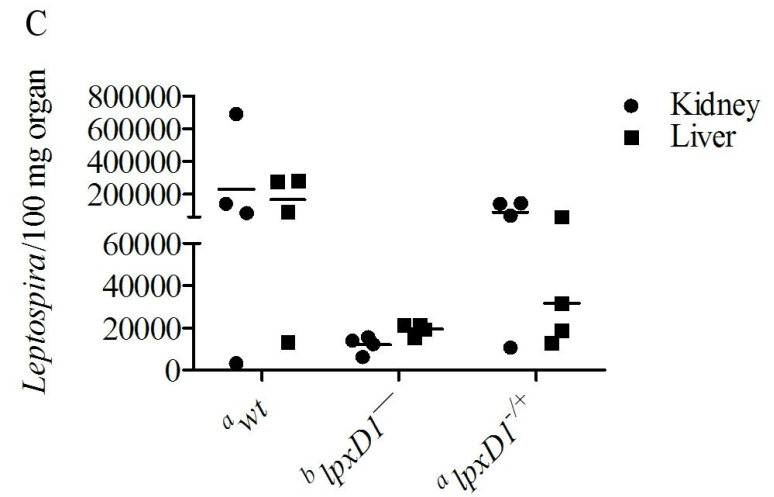
B

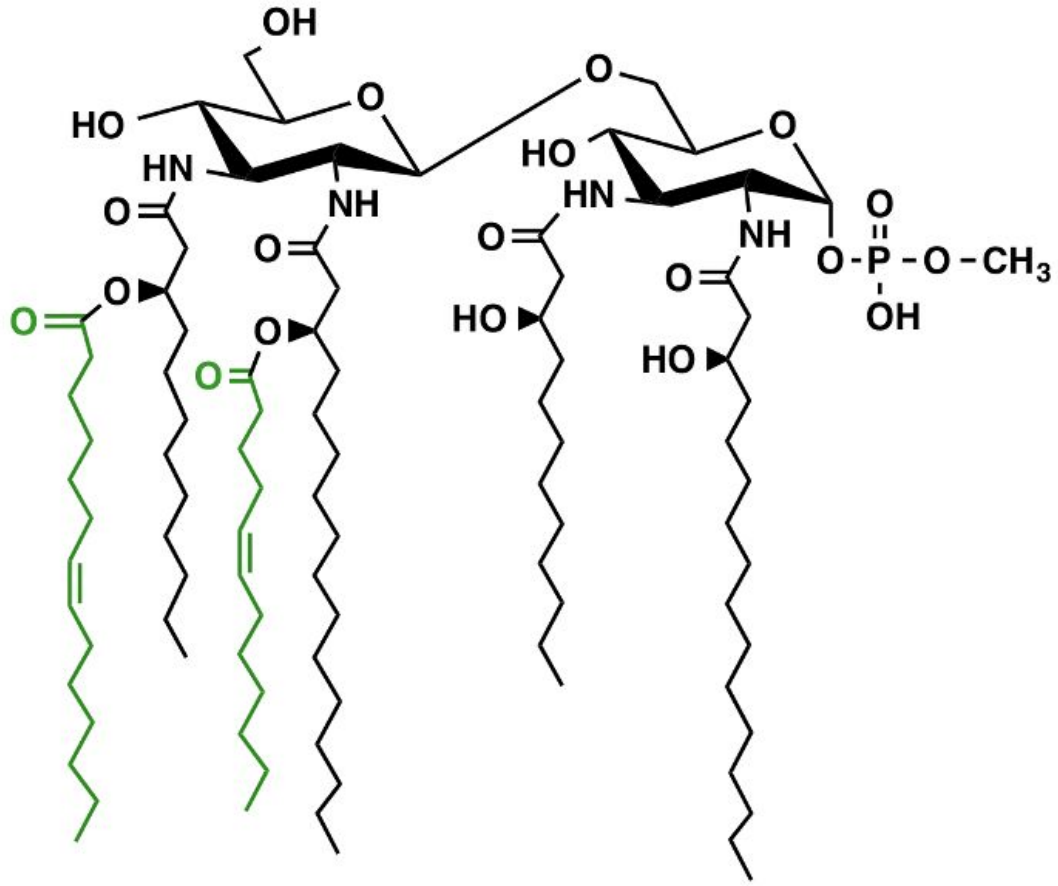


Leptospira+ serum reactivity to LPS


A
Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days
wt 10^4
lpxD1— 10^4
lpxD2— 10^4


B
Percent survival
100
80
60
40
20
0
0
5
10
15
20
Days
wt 10^4
wt 10^6
lpxD1— 10^4
lpxD1— 10^6
lpxD1—/+ 10^4
lpxD1—/+ 10^6


C
Leptospira/100 mg organ
800000
600000
400000
200000
60000
40000
20000
0
Kidney
Liver
a wt
b lpxD1—
a lpxD1-/+

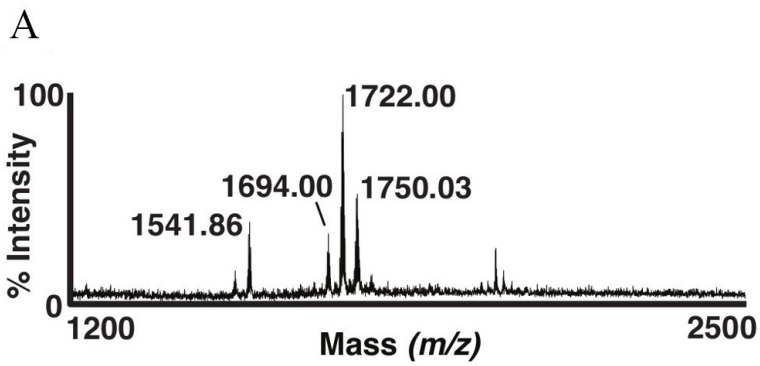


Exact Mass: 1725.27

***m/z*: 1724.27**

***m/z* with 1 more degree of unsaturation: 1722.25**

***m/z* with 2 more degrees of unsaturation: 1720.24**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100
% Intensity
0
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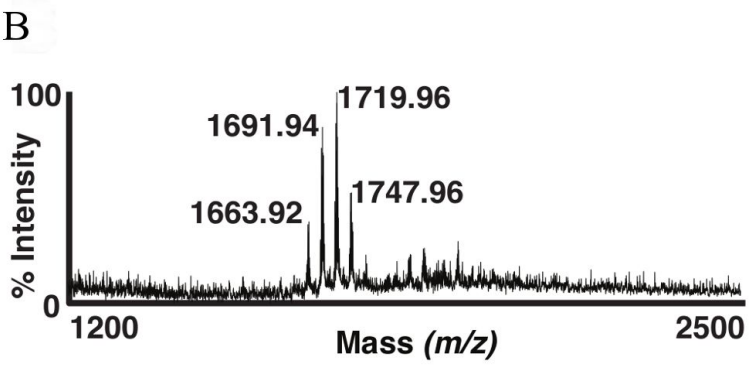
1694.00
1750.03
1541.86
1200
2500
Mass (m/z)


B
100
% Intensity
0
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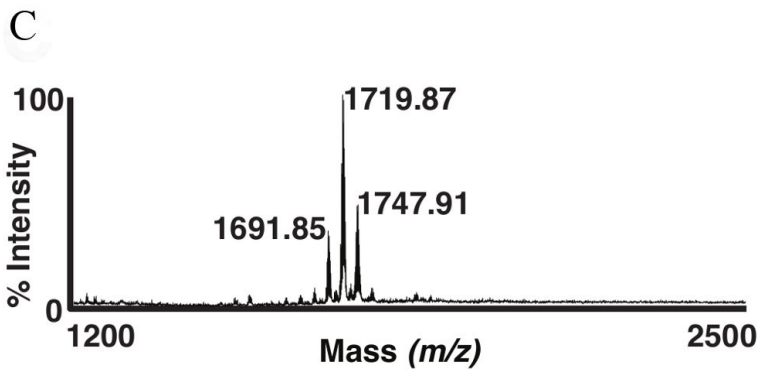
1691.94
1747.96
1663.92
1200
2500
Mass (m/z)


C
100
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1719.87
1747.91
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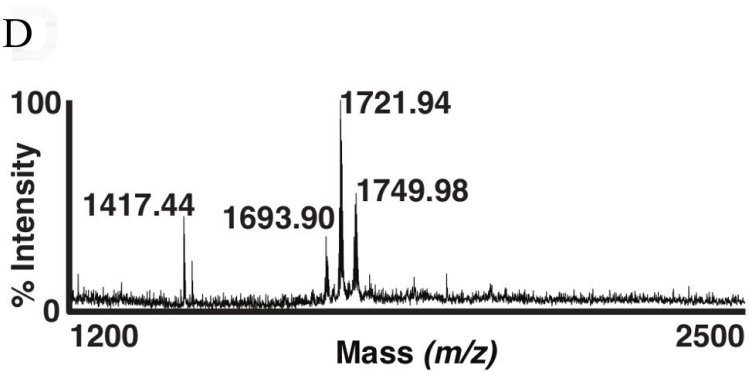
1200
2500
Mass (m/z)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100
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0
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1417.44
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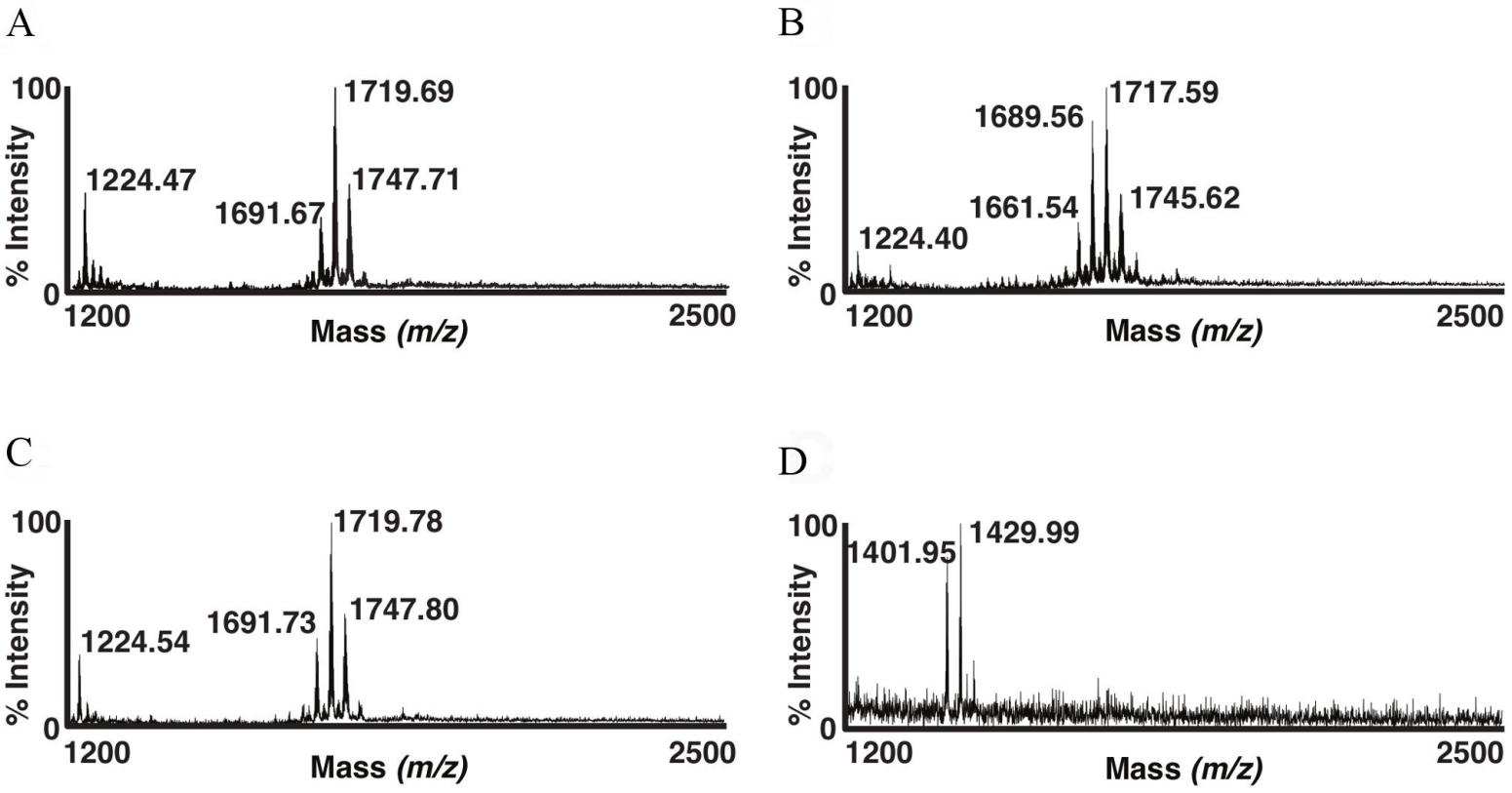
1200
2500
Mass (m/z)


A
100
% Intensity
0
1200
2500
Mass (m/z)
1719.69
1224.47
1747.71
1691.67
B
100
% Intensity
0
1200
2500
Mass (m/z)
1717.59
1689.56
1661.54
1745.62
1224.40
C
100
% Intensity
0
1200
2500
Mass (m/z)
1719.78
1224.54
1691.73
1747.80
D
100
% Intensity
0
1200
2500
Mass (m/z)
1401.95
1429.99