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Leptospiral Outer Membrane Protein LipL41 Is Not Essential for Acute Leptospirosis but Requires a Small Chaperone Protein, Lep, for Stable Expression

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Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira* spp., but knowledge of leptospiral pathogenesis remains limited. However, the development of mutagenesis systems has allowed the investigation of putative virulence factors and their involvement in leptospirosis. LipL41 is the third most abundant lipoprotein found in the outer membranes of pathogenic leptospires and has been considered a putative virulence factor. LipL41 is encoded on the large chromosome 28 bp upstream of a small open reading frame encoding a hypothetical protein of unknown function. This gene was named *lep*, for *L*ipL41 *e*xpression *p*artner. In this study, *lipL41* was found to be cotranscribed with *lep*. Two transposon mutants were characterized: a *lipL41* mutant and a *lep* mutant. In the *lep* mutant, LipL41 protein levels were reduced by approximately 90%. Lep was shown through cross-linking and coexpression experiments to bind to LipL41. Lep is proposed to be a molecular chaperone essential for the stable expression of LipL41. The roles of LipL41 and Lep in the pathogenesis of *Leptospira interrogans* were investigated; surprisingly, neither of these two unique proteins was essential for acute leptospirosis.

Pathogenic species of *Leptospira* are important and emerging zoonotic pathogens. Animal hosts of *Leptospira* species include rodents, domestic animals, and livestock (1). The bacterium is transmitted to humans through water contaminated with animal urine or through direct contact with infected animals. The disease manifests as systemic infections differing in severity, ranging from fever and flu-like symptoms to renal and liver failure, pulmonary hemorrhages, and death (2–4).

Knowledge of leptospiral pathogenesis is limited, but the development of a transposon (Tn) mutagenesis system has enabled the identification of factors essential for virulence (5), seven of which have been described: Loa22 (6), HemO (7), lipopolysaccharide (LPS) (8), motility (9, 10), ClpB (11), KatE (12), and Mce (13). Further characterization of transposon mutants will advance the understanding of leptospiral pathogenesis and biology.

The outer membrane has been a major focus for the identification of leptospiral virulence-associated factors. Outer membrane components are at the forefront of the interface between the pathogen and the host cell and thus can play a key role in adhesion and initial infection. Surface exposure, expression *in vivo*, and conservation across pathogenic species or serovars are all features indicative of potential virulence factors (14).

LipL41 is the third most abundant protein on the surfaces of pathogenic leptospires (15). It is highly conserved in pathogenic leptospiral serovars but is absent in saprophytes (16–19). LipL41 is surface exposed and is recognized by convalescent-phase human and hamster sera, indicating that it is expressed during leptospiral infection (15, 20–23). Leptospires also express LipL41 during the colonization of, and excretion from, the rat kidney (24). LipL41, along with the transmembrane protein OmpL1, forms part of a very small subset of protein antigens that have been reported to elicit protection against acute infection in hamsters (25). Interestingly, however, LipL41 alone does not confer protection (25, 26).

To date, the role of LipL41 in pathogenesis has not been evaluated. The function of LipL41 is likewise currently unknown; it has been suggested that this protein binds hemin (27), but no definitive proof of such a function has been reported.

The *lipL41* gene is located adjacent to a small gene, *la0615*, which we have designated *lep* (for *L*ipL41 *e*xpression *p*artner). In this study, we have characterized two transposon mutants, P52 (*lipL41* mutant) and M874 (*lep* mutant). We have identified Lep as a LipL41-binding protein that is essential for efficient expression of LipL41, and we have evaluated the role of both proteins in leptospiral pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* and *Leptospira interrogans* strains and the construction of transposon mutants have been described previously (5, 28). Transposon mutants were generated in *Leptospira interrogans* serovar Manilae strain L495 and in *Leptospira interrogans* serovar Pomona strain LT993 (L523). LT993 was obtained from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia. For this study, two transposon mutants with an intergenic insertion of Tn*SC189*, P19 (Tn inserted at base 430860, based on the numbering of the serovar Lai genome [19]) and M777 (29), were used as controls for transposon mutants P52 (*lipL41* mutant) and M874 (*lep* mutant), respectively. The locations of Tn*SC189* in the mutants were determined by direct sequencing of genomic DNA as described pre-

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viously (30). Strain M777, with an intergenic insertion of Tn*SC189*, has been shown previously to retain virulence (28). All *L. interrogans* strains were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson) at 30°C.

Generation of an M874 complementation construct. *lep* was amplified with primers BAP6108 (AAAAGGGCCCATGAAAAGCGGG CGAA AGT) (forward) and BAP6109 (AAAAGTCGACTCTATTTGTAGATTT TGAGAATA) (reverse), digested with restriction enzymes ApaI and KpnI, and then inserted into Tn*SC189* containing a spectinomycin resistance cassette (Tn*SC189* Spc^r). The *Borrelia burgdorferi flgB* promoter (*flgBp*) was inserted upstream of *lep*. *flgBp* was amplified with primers BAP5066 (TTAGGTACCATAATACCCGAGCTTCAAG) (forward) and BAP6107 (AAAGGGCCCATGGAAACCTCCCTCATT) (reverse) and was digested with restriction enzymes KpnI and ApaI. The *lep* complementation construct was introduced into M874 by conjugation with *E. coli*, as described previously (31).

Genome sequencing. The whole-genome shotgun (WGS) read data for *L. interrogans* serovar Manilae mutant strain M874 were determined as part of the diversity of the genome of *Leptospira interrogans* ST34 and are available under DDBJ Sequence Read Archive accession no. SRX101346. Similarly, the read data for the *L. interrogans* serovar Manilae parent strain, L495, are available under accession no. SRX101343. These read data were determined on an Illumina Genome Analyzer IIx instrument using a paired-end protocol at the J. Craig Venter Institute (JCVI, USA). Read mapping and *de novo* assembly analysis, used in the comparative analysis to examine genomic differences between these strains, were performed using SHRiMP2 (32) and Velvet (33), respectively.

SDS-PAGE and immunoblotting. Leptospires were grown to 8×10^8 cells/ml, washed twice in phosphate-buffered saline, pH 7.2 (PBS), and resuspended in sample buffer, consisting of 50 mM Tris-HCl (pH 6.8), 14.4 mM β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue in 20% glycerol, at a concentration of 1×10^9 cells/ml before serial 4-fold dilution. The whole-cell lysates (WCLs) and protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using standard methods (34). The immunoblots were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) and were probed with a rabbit antiserum against LipL41 (22) (diluted 1:2,000) or Lep (diluted 1:500) in PBS-T. The membranes were washed 3 times in PBS-T, probed with horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:5,000) (Millipore), developed using Amersham ECL Western blotting detection reagents (GE Healthcare), and exposed either to X-ray film or to a LAS-3000 chemiluminescent imager (Fujifilm). To quantify the reduction in LipL41 expression observed in M874 (*lep* mutant), the densities of the LipL41 bands in M874 and M777 WCLs from three biological replicates were compared using ImageJ (35). To confirm equal loading of the M777 and M874 WCLs, dilutions were also analyzed by Western blotting with an antiserum against LipL32 (diluted 1:4,000). Statistical analysis was performed using Student's *t* test.

Protein expression and purification. The LipL41 protein, minus the signal sequence (first 30 amino acids), was expressed in multiple cloning site 1 of pETDuet-1 with an N-terminal His tag. *lipL41* was amplified by PCR using primers BAP6247 (AAAAGGATCCGTTCCCGAAAGATAAA GAAGG) (forward) and BAP6248 (AAAAGTCGACTTTTGCGTTGCTT TCATCAAC) (reverse) and was inserted into pETDuet-1 after digestion with BamHI and SalI. The Lep protein, minus the first 25 amino acids, was expressed from multiple cloning site 2 of pETDuet-1 with a C-terminal S tag. *lep* was amplified by PCR with primers BAP6249 (AAAAAGATCTC AAAACTCCGAACGAAGAAGAA) (forward) and BAP6250 (AAAACTC GAGTTTTAATTCTTCTTTTCCGAATT) (reverse) and was inserted into pETDuet-1 after digestion with BglII and XhoI. The Loa22 protein, minus the signal sequence (first 23 amino acids), was expressed from multiple cloning site 1 of pETDuet-1 with an N-terminal His tag. *loa22* was amplified by PCR with primers BAP6591 (CTCTGCGAATTCAGCT GAAAAAAAAGAGGAATCC) (forward) and BAP6523 (TTCTAGGTCG ACTTATTGTTGTGGTGCGGAAGT) (reverse) and was inserted into pETDuet-1 after digestion with EcoRI and SalI.

Four truncations of LipL41 were generated and were expressed from multiple cloning site 1 of pETDuet-1. The PCR product of the N-terminal truncation construct LipL41Δ1-110 was amplified using primers BAP7247 (AAAAGAATTCTGGAATCACTAAAAATAGAGC) (forward) and BAP6248 (reverse). The PCR product of the N-terminal truncation construct LipL41Δ1-217 was amplified using primers BAP7249 (AAAAG AATTCTGTAGGGAATTTAGAATGTCCT) (forward) and BAP6248 (reverse). The PCR product of the C-terminal truncation construct LipL41Δ218-355 was amplified using primers BAP6247 (forward) and BAP7248 (AAAAGTCGACGCTATTGAAAATTTTCGCAGG) (reverse). The PCR products of the truncation constructs were inserted into pETDuet-1 after digestion with EcoRI and SalI. The C-terminal truncation construct LipL41Δ287-355 was generated by using an internal HindIII restriction site to remove the region corresponding to the last 68 amino acids of LipL41.

To generate an antiserum against Lep, the *lep* gene was amplified by PCR using primer BAP5772 (AAAACCATGGGAAAAACTCCGAACGA AGAA) (forward) and primer BAP5525, containing a $(His)_6$ tag (TAATC ATATGCTAGTGGTGATGATGATGTTTTGATTCTTCTTTTCC GAA) (reverse), and was inserted into pET15b after digestion with NcoI and NdeI.

All protein expression constructs were expressed in *E. coli* BL21-CodonPlus, induced with 1 to 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were disrupted by sonication, and proteins were purified from the soluble fraction. $(His)_6$-LipL41 and $(His)_6$-Loa22 were purified with Talon cobalt resin (Clontech Laboratories) according to the manufacturer's instructions. S-tagged Lep was purified with S protein agarose (Novagen, Merck) according to the manufacturer's instructions. $(His)_6$-tagged Lep was insoluble and was purified under denaturing conditions by utilizing Talon cobalt resin (Clontech Laboratories) according to the manufacturer's instructions, except that 8 M urea was used in place of 6 M guanidine-HCl. After purification, $(His)_6$-tagged Lep was dialyzed against 1 M urea. To generate an anti-Lep antiserum, two female New Zealand White rabbits were immunized intradermally with 100 μg recombinant $(His)_6$-tagged Lep in 1 M urea with 20% Alhydrogel (Sigma-Aldrich). A second and a third immunization were given after 2 and 4 weeks, and the rabbits were bled 2 weeks later. Rabbit experiments were approved by the Monash University Animal Ethics Committee.

Cross-linking of leptospiral cells. Leptospiral cells were grown to a density of 5×10^8/ml, pelleted at $4,000 \times g$, and washed twice with PBS, pH 8. The cells were cross-linked with 5 mM dimethyl 3,3′-dithiobispropionimidate dihydrochloride (DTBP) (Sigma-Aldrich) and were incubated on ice for 1 to 2 h. The cross-linking reaction was quenched by the addition of Tris-HCl, pH 8, at a final concentration of 50 mM and incubation on ice for a further 10 min. The cells were disrupted by sonication and were analyzed by SDS-PAGE and immunoblotting. To reduce the background, prior to anti-Lep immunoblotting, the cross-linked samples and whole-cell lysates were separated by SDS-PAGE, and the gel region corresponding to 64 kDa was then excised from each sample, separated by SDS-PAGE, and probed with the IgG fraction of anti-Lep antiserum, purified by using protein A-Sepharose beads (GE Healthcare) according to the manufacturer's instructions.

Triton X-114 extraction. Outer membrane proteins were extracted with Triton X-114 as described previously (36). After extraction, the detergent and aqueous phases were precipitated with methanol-chloroform.

Hemin binding. Hemin binding experiments were performed using hemin agarose (type I; Sigma-Aldrich). Wild-type and *lipL41* mutant P52 cells were grown to mid-log phase; 10^9 cells were pelleted and were washed twice with Tris-buffered saline, pH 7.4. The cells were resuspended in 25 mM Tris-HCl and 100 mM NaCl, pH 7.4, and were disrupted by sonication; insoluble material was removed by centrifugation at $13,000 \times g$ for 5 min. One hundred microliters of hemin agarose was washed twice with 25 mM Tris-HCl and 100 mM NaCl, pH 7.4, and the beads were pelleted at

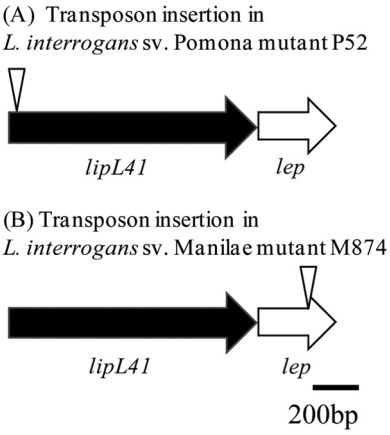

FIG 1 Locations of transposon insertions in *L. interrogans* mutants of serovar (sv.) Pomona (A) and serovar Manilae (B) strains. Vertical arrowheads indicate Tn*SC189* insertions. Tn*SC189* was inserted 42 bp into the open reading frame of *lipL41* (1,068 bp) in P52 and 232 bp into the open reading frame of *lep* (333 bp) in M874.

800 × *g* for 5 to 10 min before incubation with the leptospiral cell lysates at 37°C for 1 to 2 h with gentle agitation. The unbound proteins were removed, and the hemin agarose was washed six times as described above. The hemin agarose was resuspended in sample buffer, boiled for 10 min, and analyzed by SDS-PAGE and anti-LipL41 immunoblotting.

Spectral analysis of the hemin-LipL41 complex was performed as described previously (30). Briefly, 24 μM bovine hemin (Sigma) was added to 0.7 μM purified recombinant LipL41 in 5-μl increments. The absorbance spectrum was measured after each addition of hemin.

Infection of hamsters. *L. interrogans* serovar Manilae strains L495 and M874 and *L. interrogans* serovar Pomona strains L523 and P52 were tested for virulence in hamsters. Groups of 10 golden hamsters of either sex, aged 4 to 6 weeks, were infected intraperitoneally with 10^3 leptospires of serovar Manilae strains (50% infective dose [ID_{50}], <10 leptospires) or 10^4 leptospires of serovar Pomona strains (ID_{50}, <100 leptospires) in 100 μl EMJH medium and were monitored for 21 days. Moribund animals were euthanized in accordance with animal ethics requirements. Lung hemorrhages were assessed, and kidney tissue was collected postmortem for culture. Hamster experiments were approved by the Khon Kaen University Animal Ethics Committee.

RESULTS

Bioinformatic analysis of *lipL41* and *lep*. LipL41 (355 amino acids) is highly conserved within pathogenic *Leptospira* spp.; full-length LipL41 proteins share ≥96% identity within *L. interrogans* and *Leptospira borgpetersenii* serovars. LipL41 bears little sequence similarity to other leptospiral proteins; the closest match is LA2873 (*L. interrogans* serovar Lai), a putative fructose-1,6-bisphosphatase (23% identity for residues 179 to 340). Outside of the genus *Leptospira*, LipL41 shows very little similarity to other prokaryotic proteins; the closest is the hypothetical protein Lepil_1901 from *Leptonema illini*, a member of the family *Leptospiraceae*, which shares 29% identity with LipL41 over the length of the protein (residues 3 to 327).

Within the leptospiral genome, *lipL41* is located in a putative operon 28 bp upstream of the small gene *lep* (333 bp) (Fig. 1). Both the *lep* gene and its genomic arrangement with *lipL41* are highly conserved among *L. interrogans* and *L. borgpetersenii* serovars (≥87% identity) (16, 17, 19). Within the genus *Leptospira*, the closest homolog to Lep is another hypothetical protein, LA2279 (*L. interrogans* serovar Lai), sharing 29% identity (residues 36 to 93 of Lep). Like LipL41, Lep shows little similarity to other bacterial proteins. The most similar protein outside the genus *Leptospira* is a hypothetical protein from the marine myxobacterium *Plesiocystis pacifica*; 27% identity is observed from residue 13 to 91 of Lep. No conserved domains have been identified for either LipL41 or Lep, and their functions remain unclear. Neither Lep nor LipL41 is present in the saprophyte *Leptospira biflexa*; thus, these proteins are unique to pathogenic leptospiral species. Bioinformatic analysis was performed using the protein basic local alignment search tool (BLAST) (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Inactivation of *lep* results in reduced LipL41 protein expression. This study utilized two *L. interrogans* transposon mutants, P52 (serovar Pomona *lipL41* mutant) and M874 (serovar Manilae *lep* mutant). These mutants were generated by random transposon mutagenesis as described previously (5, 28). Tn*SC189* was inserted 42 nucleotides (nt) (corresponding to 14 amino acid residues of 355) from the start of *lipL41* in P52, while in M874, Tn*SC189* was inserted 232 nt (77 amino acid residues of 110) from the start of *lep* (Fig. 1). In cellular morphology, growth, and motility, P52 and M874 were indistinguishable from the corresponding wild-type strains.

The disruption of *lipL41* and *lep* by transposon insertion is unlikely to affect the transcription of genes surrounding the *lipL41-lep* locus, since neighboring genes are transcribed in the direction opposite to that of the locus. The hypothetical gene *la0617* is located 115 bp upstream of *lipL41*, and a putative *ftsZ* gene (*la0612*) is located 476 bp downstream of *lep*.

Protein immunoblot analysis was performed with anti-LipL41 and anti-Lep sera on whole-cell lysates (WCLs) of P52 and M874, and their respective wild-type controls, to confirm the phenotype of each mutant (Fig. 2A). P52 did not express LipL41 or Lep, while M874 did not express Lep. The *lep* mutant M874 showed reduced expression of LipL41. To quantify the level of LipL41 expression in M874, leptospiral WCLs were serially diluted and were analyzed by immunoblotting with an anti-LipL41 serum (Fig. 2B). Anti-LipL32 immunoblots served as loading controls (Fig. 2B). The *lep* mutant M874 showed significantly reduced LipL41 expression, producing only 12% of the amount of LipL41 found in the wild type (Fig. 2B). Total-membrane fractions of wild-type and M874 cells were also analyzed by 2-dimensional gel electrophoresis twice, in biological duplicate. No differentially expressed proteins other than LipL41 were observed; immunoblot analysis confirmed the reduction in LipL41 expression (data not shown).

The proximity of *lipL41* and *lep* on the leptospiral genome suggested that they are cotranscribed; hence, the insertion of Tn*SC189* in *lep* may affect the transcription of *lipL41* by destabilizing the *lipL41* transcript. The levels of *lipL41* and *lep* transcripts were analyzed by semiquantitative reverse transcriptase PCR (RT-PCR). *lipL41*-, *lep*-, and gene junction-specific products were amplified from M874 and wild-type cDNA by PCR. There was no difference in the amounts of *lipL41*-, *lep*-, and gene junction-specific products (data not shown), indicating that *lipL41* and *lep* are cotranscribed and that the reduction in LipL41 expression in M874 was not due to reduced transcription of *lipL41*.

Attempted complementation and genome sequence of the *lep* mutant. Numerous attempts were made to complement M874 with Tn*SC189* containing a spectinomycin resistance cassette and full-length *lep*. Spectinomycin-resistant transformants with an in-

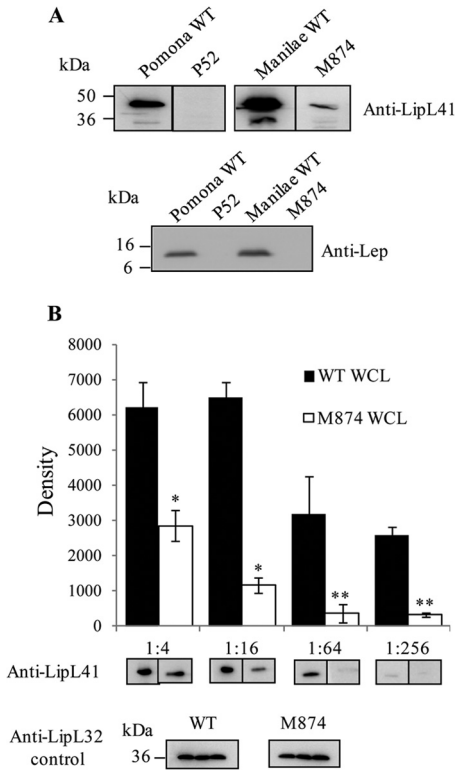

FIG 2 (A) Immunoblot analysis of a wild-type (WT) serovar Pomona strain, P52, a WT serovar Manilae strain, and M874 with anti-LipL41 and anti-Lep antisera. The positions of molecular mass markers (in kilodaltons) are indicated on the left. (B) Quantification of LipL41-specific bands in WCLs of the *lep* mutant M874 and the WT serovar Manilae strain. Bacterial WCLs were serially diluted, separated by SDS-PAGE, and analyzed by immunoblotting with an anti-LipL41 antiserum. Means from three experiments are presented. Error bars represent standard errors of the means ($n = 3$). Asterisks indicate significant differences by Student's t test (*, $P < 0.05$; **, $P < 0.01$). Anti-LipL32 immunoblots were used as loading controls for undiluted samples.

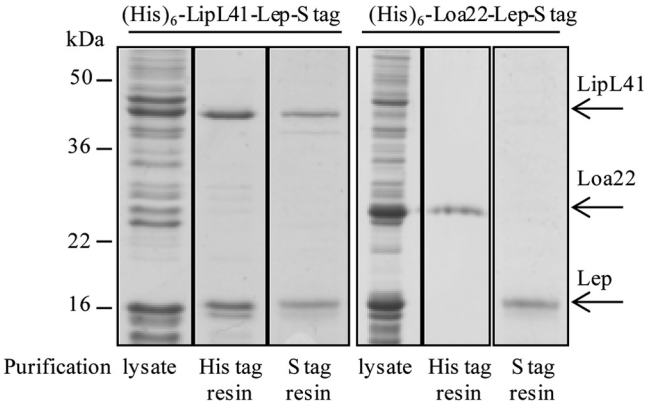

FIG 3 Copurification of LipL41 and Lep by affinity resin purification. Coexpressed recombinant proteins LipL41 and Lep, or Loa22 and Lep, were purified by metal affinity resin (His tag resin) or S protein resin (S tag resin) and were separated on a Coomassie blue-stained 12% SDS-polyacrylamide gel. Lysate, soluble fraction of whole-cell lysates; His tag resin, fraction purified using metal affinity resin; S tag resin, fraction purified using S protein resin. The positions of protein molecular mass markers (in kilodaltons) are indicated on the left. The locations of LipL41, Loa22, and Lep are indicated by arrows on the right.

sertion of Tn*SC189* containing *lep* were obtained; however, no expression of Lep could be detected by immunoblotting with an anti-Lep antiserum. Therefore, the restoration of LipL41 protein expression could not be evaluated.

In the absence of complementation, the genome sequence of M874 was compared to that of the serovar Manilae parent strain. Paired-end WGS read data comprising 628.7 million bases, representing ~140-fold coverage of the genome, for the parent strain (L495) and 834 million bases, representing ~180-fold coverage of the genome, for M874 were used to examine differences between the genomes of these strains. The disruption of *lep* by Tn*SC189* was confirmed in M874, but comparative analysis of the genome sequence revealed no additional genomic rearrangements, deletions, or point mutations, confirming that the reduction of LipL41 expression was due to inactivation of the *lep* gene.

LipL41 and Lep proteins interact. The reduced LipL41 levels in the *lep* mutant and the cotranscription of *lipL41* and *lep* indicated a potential for interaction between the two proteins; Lep may act as a chaperone conferring stability on LipL41, which would be degraded in the absence of Lep. To assess protein-protein interactions, recombinant LipL41 and Lep were generated. LipL41 was initially expressed in pET28a with an N-terminal His tag but was found to be largely insoluble (data not shown). The expression of recombinant proteins with a chaperone or binding partner can increase their solubility (37, 38). Accordingly, *lipL41* and *lep* were cloned into the pETDuet-1 expression vector, which allows the coexpression of proteins from independent plasmid sites with different protein tags. When LipL41 (with an N-terminal His tag) was coexpressed from pETDuet-1 with Lep (with a C-terminal S tag), LipL41 exhibited increased solubility, suggesting a stabilizing interaction between the two proteins.

Metal affinity resin was used to purify $(His)_6$-LipL41 that was coexpressed with Lep. Purification of $(His)_6$-LipL41 resulted in the copurification of S-tagged Lep (Fig. 3). In the reverse experiment, S-tagged Lep was purified on the S protein resin, and $(His)_6$-LipL41 was found to copurify (Fig. 3). The copurification of LipL41 and Lep via the two different tags provides evidence that LipL41 and Lep interact. As a negative control for the copurification of LipL41 and Lep, Loa22, a leptospiral OmpA-like protein (6), was expressed from pETDuet-1 together with Lep. When coexpressed with S-tagged Lep, $(His)_6$-Loa22 was purified using metal affinity resin and did not copurify with Lep. Likewise, no copurification was observed between $(His)_6$-Loa22 and S-tagged Lep when the S protein resin was utilized (Fig. 3). The absence of copurification for Loa22 and Lep confirmed the specificity of the protein-protein interaction between LipL41 and Lep.

To further characterize the interaction between LipL41 and Lep, protein cross-linking was performed on leptospiral cells. The cross-linker dimethyl 3,3′-dithiobispropionimidate (DTBP), a membrane-permeant imidoester, was used to cross-link proteins that are associated or are in close proximity to each other in P52 (*lipL41* mutant), M874 (*lep* mutant), and the wild-type strains. Immunoblotting with an anti-LipL41 serum (Fig. 4A) showed that in the wild-type strains, the monomeric form of LipL41 migrated at approximately 38 kDa. A LipL41-containing band, at an abundance lower than that of monomeric LipL41, was also detected, at approximately 64 kDa, in wild-type strains treated with the cross-linker DTBP (Fig. 4A); it was not present in the absence of cross-linking. This complex was not detected in P52 (*lipL41*

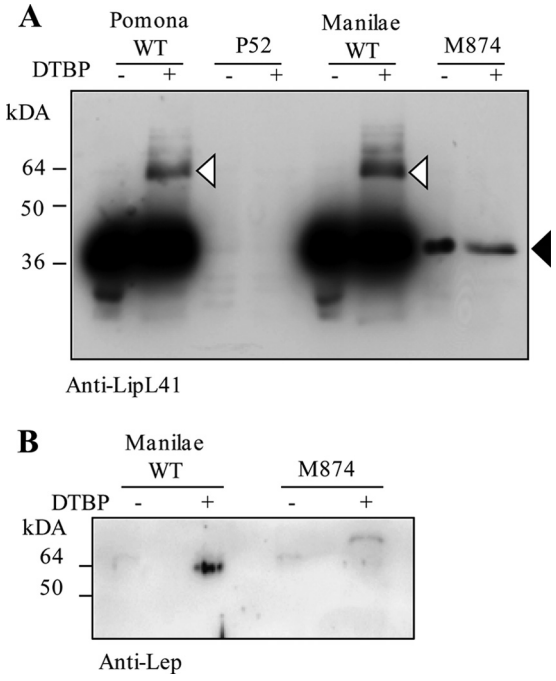

FIG 4 Cross-linking of *L. interrogans* probed with an anti-LipL41 or anti-Lep antiserum. (A) A serovar Pomona wild-type (WT) strain, P52 (*lipL41* mutant), a serovar Manilae WT strain, and M874 (*lep* mutant) were treated with the cross-linker DTBP (dimethyl 3,3′-dithiobispropionimidate), and the lysates were analyzed by immunoblotting with an anti-LipL41 antiserum. The filled arrowhead indicates LipL41 at 38 kDa; open arrowheads indicate the LipL41 complex at 64 kDa. +, DTBP-treated cells; −, cells not treated with DTBP. The positions of protein molecular mass markers (in kilodaltons) are indicated on the left. (B) WCLs of the serovar Manilae WT strain or the *lep* mutant M874, either left untreated or treated with DTBP, were analyzed by anti-Lep immunoblotting. Anti-Lep antibodies recognize a 64-kDa complex only in the wild-type strain treated with DTBP.

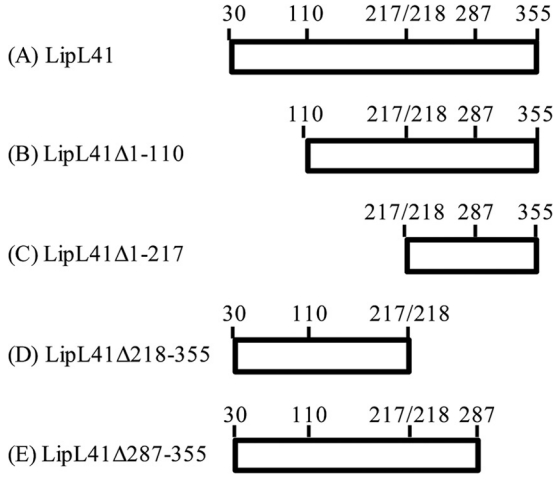

FIG 5 Schematic representation of recombinant LipL41 and LipL41 truncation constructs expressed in pETDuet-1 with Lep. (A) Full-length LipL41 (expressing amino acids 30 to 355); (B) LipL41Δ1-110 (amino acids 111 to 355); (C) LipL41Δ1-217 (amino acids 218 to 355); (D) LipL41Δ218-355 (amino acids 30 to 217); (E) LipL41Δ287-355 (amino acids 30 to 287).

mutant). The 64-kDa LipL41 complex band was likewise not detected in M874 (*lep* mutant), in agreement with the notion that the complex contains Lep. To confirm the presence of Lep in the 64-kDa LipL41 complex, anti-Lep immunoblotting was performed. Non-cross-linked and cross-linked WCLs of the wild-type serovar Manilae strain and the *lep* mutant M874 were separated by SDS-PAGE. The region on the gel corresponding to 64 kDa was excised, separated by SDS-PAGE a second time, and probed with an anti-Lep antiserum (Fig. 4B.) A 64-kDa band was observed only in the wild-type cells treated with the cross-linker DTBP, confirming the presence of Lep in the LipL41 complex. The size of the LipL41-Lep complex, at 64 kDa, corresponds to 1 LipL41 protein unit (approximately 38 kDa) and 2 Lep protein units (approximately 13 kDa each).

Lep interacts predominantly with the N terminus of LipL41. To identify the region of LipL41 involved in binding to Lep, four truncation constructs of LipL41 were coexpressed with Lep in pETDuet-1 (Fig. 5). The LipL41 truncation constructs were His tagged and were purified with metal affinity resin. LipL41 mutants deficient in the N-terminal region (LipL41Δ1-110 and LipL41Δ1-217) showed levels of Lep copurification lower than those of full-length LipL41 and the fragments in which the N terminus was present (LipL41Δ218-355 and LipL41Δ287-355) (Fig. 6A).

The amounts of LipL41 and Lep purified were quantified and standardized for the molecular masses of the purified proteins (Fig. 6B). Two Lep molecules were purified for each full-length LipL41 molecule; this ratio is consistent with the 64-kDa complex observed in leptospiral cells after cross-linking. This purification ratio was also observed for LipL41 truncation constructs LipL41Δ218-355 and LipL41Δ287-355. The copurification of Lep was not completely abolished by the removal of the N-terminal region of LipL41; however, the ratio of Lep units to LipL41 units purified was significantly reduced, to 0.5 (Fig. 6B).

Lep does not affect the trafficking of LipL41 to the outer membrane. The LipL41-binding properties of Lep highlight it as a LipL41 chaperone; however, the specific role of Lep is unclear. The cellular location of Lep and its involvement in the trafficking of LipL41 to the outer membrane were therefore evaluated. The proportions of LipL41 in the outer membrane and cytoplasm were analyzed. Quadruplicate wild-type and M874 cultures were treated with Triton X-114, and the cytoplasmic and outer membrane fractions (aqueous and detergent-extracted fractions, respectively) were analyzed by immunoblotting. LipL41 localized to the detergent fraction, as reported previously (22), a common feature of leptospiral outer membrane lipoproteins. The proportion of LipL41 in each fraction was the same for the wild type and the *lep* mutant (approximately 50% in each fraction) (Fig. 7A), indicating that the loss of Lep in M874 did not affect the trafficking of LipL41 to the outer membrane. Analysis of Triton X-114-extracted cell fractions with an anti-Lep serum showed that Lep was found exclusively in the cytoplasmic fractions of wild-type cells (Fig. 7B). The quality of the Triton X-114 cell fractionation was verified by immunoblotting with antisera against LipL21 (an outer membrane protein) and LipL31 (a cytoplasmic membrane protein); the results indicated a clean separation, with no cross-contamination of fractions (Fig. 7B).

Does LipL41 bind hemin? The function of LipL41 remains unknown. A previous study using hemin-linked agarose beads proposed that LipL41 had hemin-binding properties (27); however, this proposal was presented with limited evidence. We investigated the hemin-binding capacity of LipL41 with hemin agarose,

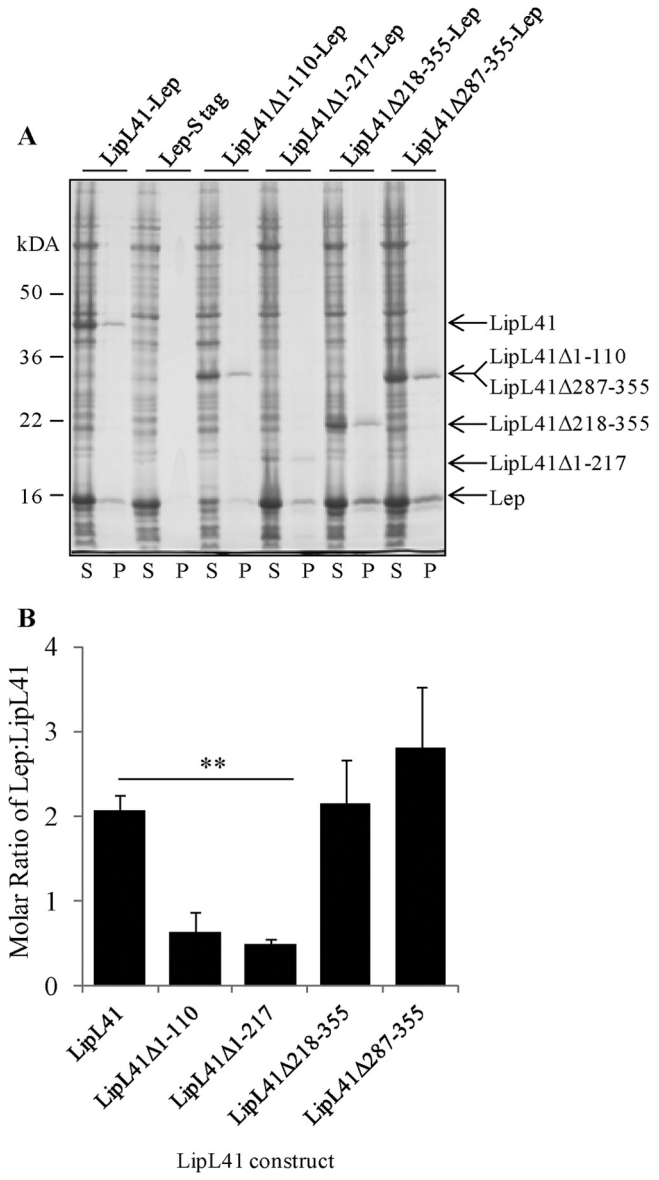

FIG 6 (A) Coomassie blue-stained 12% SDS-polyacrylamide gel of soluble lysates (S) and purified fractions (P) of recombinant LipL41 and LipL41 truncation constructs coexpressed with S-tagged Lep. Purifications were performed with metal affinity resin. (B) Molar ratio of purified Lep to purified LipL41, based on densitometry analysis of bands. The error bars represent standard errors of the means ($n = 3$). Asterisks indicate significant differences (**, $P < 0.01$) by Student's *t* test.

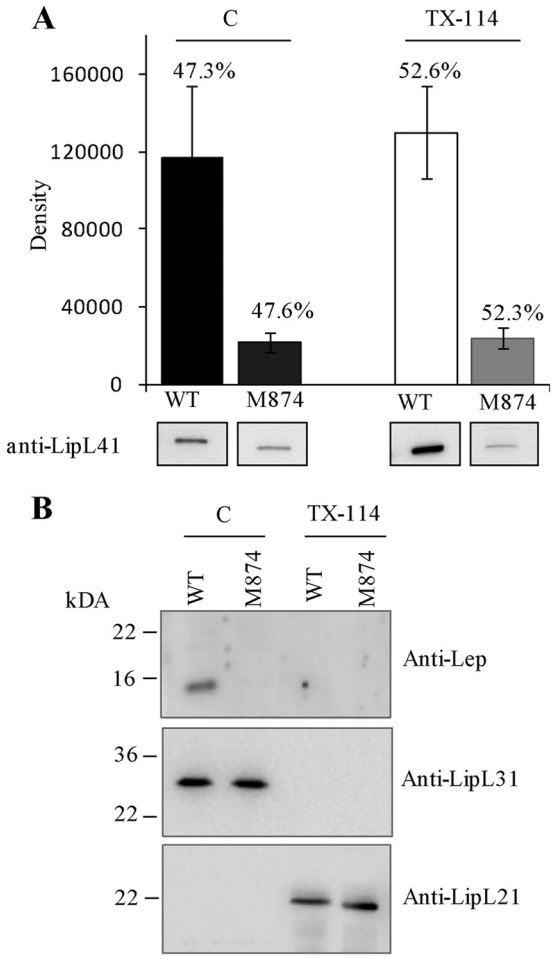

FIG 7 Analysis of the subcellular distribution of LipL41 in the wild type and M874 (*lep* mutant) by Triton X-114 extraction. (A) Wild-type and M874 cytoplasmic fractions (C) and detergent fractions (TX-114) were analyzed by anti-LipL41 immunoblotting, and the densities of the bands were quantified. The proportion of LipL41 from each strain represented in each cellular fraction is given above the bars on the graph. Error bars represent standard errors of the means ($n = 4$). (B) Wild-type and M874 cytoplasmic and detergent fractions were also analyzed with anti-Lep, anti-LipL31, and anti-LipL21.

using a method similar to that described by Asuthkar et al. (27). Cell lysates of the wild-type serovar Pomona strain and *lipL41* mutant P52 were incubated either with hemin agarose or with agarose beads without hemin. LipL41 immunoblot analysis revealed that LipL41 bound agarose in the absence of hemin. We also performed spectral analysis of the binding between recombinant LipL41 and hemin, as described by Murray et al. (30), and again found no evidence of hemin binding by LipL41. Our finding that LipL41 has an affinity for agarose beads (data not shown) could explain the observations made by Asuthkar et al. (27).

LipL41 and Lep are not essential for virulence in hamsters. LipL41 is the third most abundant lipoprotein unique to pathogenic leptospires and has a number of features that suggest a role in leptospiral pathogenesis. Until now there has been no evaluation of the role that LipL41 plays in acute leptospirosis. P52 (*lipL41* mutant) and M874 (*lep* mutant) were evaluated for virulence in the hamster model of acute disease. Groups of 10 hamsters were inoculated intraperitoneally with either P52, M874, the wild-type serovar Pomona strain, or the wild-type serovar Manilae strain. The serovar Manilae intergenic mutant M777 was not included in this experiment but has been shown previously to be virulent (28). P52 and M874 were not attenuated: all animals succumbed to infection (Table 1). Macroscopic lung hemorrhages occurred with similar frequencies and severities in animals infected with mutant or wild-type leptospires. Leptospires were cultured from the kidneys of 8 of 10 hamsters infected with P52 and from the kidneys of all hamsters infected with M874. The genotypes of reisolated mutants were confirmed by PCR, thus confirming the retention of the transposon.

TABLE 1 Survival of hamsters infected with control or mutant strains of *L. interrogans* serovar Manilae or *L. interrogans* serovar Pomona

Strain or medium	Description	Dose	Survival rate[a]
Wild-type serovar Pomona strain LT993	Parent strain	10^4	0/10
P52	*lipL41* mutant	10^4	0/10
Wild-type serovar Manilae strain L495	Parent strain	10^3	0/10
M874	*lep* mutant	10^3	0/10
EMJH medium	Negative control		10/10

[a] Given as the number of animals surviving/total number of animals in the group. The survival of animals infected with mutants was not significantly different (by Fisher's exact test) from the survival of those infected with the corresponding control strains.

DISCUSSION

The pathogenesis of leptospirosis remains poorly understood, and until the development of mutagenesis systems, it was extremely difficult to study the factors essential for this zoonotic pathogen to cause disease (14). LipL41 is a major outer membrane lipoprotein, and its high abundance, surface exposure, and exclusivity to pathogenic *Leptospira* species have made it an attractive putative virulence factor. In this study, we have characterized two *L. interrogans* transposon mutants: P52 (serovar Pomona), a *lipL41* mutant, and M874 (serovar Manilae), a *lep* mutant. We found that *lipL41* and *lep* are cotranscribed and that, as a consequence, P52 does not express either protein. Interestingly, we found that disruption of *lep* resulted in reduced LipL41 protein expression but did not affect the transcription of *lipL41*.

We were unable to complement M874. Insertion of Tn*SC189* containing full-length *lep* was achieved; however, no expression of the protein was detected by immunoblotting. We were therefore unable to assess the restoration of LipL41 expression. Importantly, however, genome sequence analysis of M874 confirmed the disruption of *lep* by Tn*SC189* and found no other mutations to account for the reduction in LipL41 protein expression in M874.

We hypothesized that the LipL41 and Lep proteins interact, with Lep acting as a chaperone to stabilize LipL41 expression. We have presented several lines of evidence to support this hypothesis: (i) coexpression of Lep with LipL41 increased the solubility of LipL41; (ii) copurification of the proteins by affinity purification revealed that LipL41 and Lep interact; (iii) cross-linking experiments showed that LipL41 and Lep form a complex of approximately 64 kDa, corresponding to the mass of 1 LipL41 molecule and 2 Lep molecules. The interaction between LipL41 and Lep appears to be transient due to the low abundance of the complex, consistent with the finding that Lep is restricted to the cytoplasm whereas LipL41 is trafficked to the outer membrane. The same ratio of 2 Lep units to 1 LipL41 unit was also observed during the copurification of recombinant proteins.

Many characterized bacterial chaperones have discrete regions for binding within the substrate (39–41); we observed a similar phenomenon for binding between LipL41 and Lep, where removal of the N terminus of LipL41 resulted in reduced interaction with Lep. The loss of Lep in M874 did not impact on the trafficking of LipL41 to the outer membrane; cell fractionation revealed that wild-type and M874 (*lep* mutant) cells had similar proportions of LipL41 both in the cytoplasmic fractions and in the outer membrane fractions.

Another common feature of characterized chaperones is their requirement for stable expression of the substrate, such as the chaperone SseA of *Salmonella* spp., which is required for stable expression of SseB and SseD (42). This was also observed for Lep and LipL41. Lep exhibits many features that are typical of characterized chaperones, including cytoplasmic location, small size (<15 kDa), and the property of being functionally active as a dimer (42–45). All these features of Lep are consistent with its role as a cytoplasmic chaperone stabilizing LipL41 expression.

The observed increase in LipL41 solubility with the coexpression of Lep is indeed interesting. *Leptospira* species have a large number of membrane lipoproteins, which, when expressed in *E. coli*, can be insoluble. The coexpression of other leptospiral membrane proteins with Lep, or with other chaperones, may help to increase the solubility and facilitate the purification and characterization of these membrane proteins.

This study showed, for the first time, that the major outer membrane protein LipL41 is not required for leptospiral virulence in the hamster model of acute leptospirosis. All the animals succumbed to infection, and lung hemorrhages were observed at the same frequency and severity in animals infected with mutant or wild-type leptospires. *lipL41* and *lep* mutant leptospires also colonized the kidney. The *lep* mutant also retained the ability to colonize mouse kidneys in a carrier model of leptospirosis (R. A. Marcsisin, T. Bartpho, D. M. Bulach, A. Srikram, R. W. Sermswan, B. Adler, and G. L. Murray, submitted for publication). This result for M874 is in contrast with the findings of a previous preliminary study using small numbers of animals, which suggested that the *lep* (LA0615) transposon mutant might be attenuated in hamsters (28). The results presented in the present study clearly show that Lep is not required for acute leptospirosis in the hamster model.

It is surprising that the *lipL41* mutant retained virulence; many features of LipL41 and its expression *in vivo* strongly suggested involvement in pathogenesis. However, similar results have been reported for LipL32 and LigB; both proteins bear all the hallmarks of virulence factors, including outer membrane location, expression *in vivo*, conservation in pathogenic leptospires, and demonstrated binding to host proteins *in vitro*, but like LipL41, both LipL32 and LigB are not required for virulence in hamsters or for the colonization of rat kidneys (29, 46). All of these results are consistent with a high degree of functional redundancy in leptospiral virulence genes (1).

The function of LipL41 remains unknown. A previous study proposed that LipL41 had hemin-binding properties (27); however, we observed no hemin binding by LipL41. Although we have shown that LipL41 is not essential for acute leptospirosis, it may still play an important role in pathogenic *Leptospira* species. One study has found downregulation of LipL41 during interaction with macrophage-derived cells (47). However, other studies have shown that LipL41 is expressed during acute infection, colonization of kidneys, and excretion from host kidneys (24, 48). The characterization of leptospiral mutants is fundamental to the understanding of the virulence factors of this global pathogen. It enables better understanding of leptospiral pathogenesis and allows leptospiral research to move toward more-efficient prevention and treatment strategies. We have shown in this study that the major outer membrane lipoprotein LipL41 is not essential for virulence. The function of this protein is still undetermined, but the expression of LipL41 is reliant on a small, novel chaperone protein, Lep.

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