Leptospiral protein antigens are of interest as potential virulence factors and as candidate serodiagnostic and immunoprotective reagents. We identified leptospiral protein antigens by screening a genomic expression library with serum from a rabbit hyperimmunized with formalin-killed, virulent *Leptospira kirschneri* serovar *grippotyphosa*. Genes expressing known outer membrane lipoproteins LipL32 and LipL41, the heat shock protein GroEL, and the α, β, and β′ subunits of RNA polymerase were isolated from the library. In addition, a new leptospiral gene that in *Escherichia coli* expressed a 45-kDa antigen with an amino-terminal signal peptide followed by the spirochetal lipobox Val→Phe→Asn→Ala→Cys→ was isolated. We designated this putative lipoprotein LipL45. Immunoblot analysis of a panel of *Leptospira* strains probed with LipL45 antisem demonstrated that many low-passage strains expressed LipL45. In contrast, LipL45 was not detected in high-passage, culture-attenuated strains, suggesting that LipL45 is a virulence-associated protein. In addition, all leptospiral strains tested, irrespective of culture passage, expressed a 31-kDa antigen that was recognized by LipL45 antisem. Southern blot and peptide mapping studies indicated that this 31-kDa antigen was derived from the carboxy terminus of LipL45; therefore, it was designated P31LipL45. Membrane fractionation studies demonstrated that P31LipL45 is a peripheral membrane protein. Finally, we found that P31LipL45 levels increased as *Leptospira* entered the stationary phase, indicating that P31LipL45 levels were regulated. Hamsters infected with *L. kirschneri* formed an antibody response to LipL45, indicating that LipL45 was expressed during infection. Furthermore, the immunohistochemistry of kidneys from infected hamsters indicated that LipL45 was expressed by *L. kirschneri* that colonized the renal tubule. These observations suggest that expression of LipL45 responds to environmental cues, including those encountered during infection of a mammalian host.

Spirochetes of the genus *Leptospira* are the causative agents of leptospirosis, an emerging zoonosis encountered worldwide. Many animals serve as reservoir hosts and transmit the pathogen directly or indirectly to humans. Leptospires persist in the proximal renal tubules of kidneys in reservoir hosts and are shed in the urine into the surrounding environment. Leptospires can survive for weeks in soil or water before they are acquired by a new host via abrasions or by penetration of mucous membranes. Human victims of leptospirosis present with symptoms that vary in severity from mild flu-like symptoms to lethal pulmonary hemorrhage or hepatorenal failure (36).

At least 268 serovars and 17 species of *Leptospira* and *Leptonema* have been described to date (9). The antigenic basis for serovar specificity resides in the carbohydrate side chain structure of lipopolysaccharides (LPS) (12). Antibodies targeting leptospiral LPS provide protective immunity (32, 33). Because of the variety of LPS forms presented to the host immune system by different serovars during leptospirosis, the currently available whole-cell vaccines do not provide cross-protection against infection by heterologous serovars. Moreover, in some cases, these vaccines may not even protect against homologous challenge (6). New vaccine strategies are necessary to provide effective protection against the diversity of serovars that a susceptible host may encounter.

A recent study showed that an LPS-depleted outer membrane protein extract provided hamsters with complete protection against homologous leptosporal challenge and partial protection against heterologous lethal challenge (49). The protection appeared to be protein mediated since LPS from the same organism did not protect hamsters from lethal infection by a heterologous serovar (49). These results suggest that an effective subunit vaccine can be developed by using one or more leptospiral proteins. Proteins located in the leptospiral outer membrane are of the greatest interest in this regard because outer membrane proteins are potentially exposed to the host immune system on the leptospiral surface. Antigenic conservation of leptospiral proteins was demonstrated in immunoblot studies that showed that sera from rabbits hyperimmunized with one strain recognized numerous proteins of a wide variety of serovars (11, 39). Several of these protein antigens have been identified, including the outer membrane lipoproteins LipL32 (24) and LipL41 (45) and the porin...
OmpL1 (23), as well as the heat shock proteins DnaK (2) and GroEL (3). In an initial study to examine the feasibility of using outer membrane proteins as subunit vaccines, recombi-
nant OmpL1 and LipL41 expressed as Escherichia coli mem-
brane proteins synergistically protected Golden Syrian ham-
sters from homologous challenge (26). Although the hamsters were not challenged with heterologous serovars of Leptospira in this study, the antigenic conservation of LipL41 and OmpL1 among all pathogenic serovars of Leptospira suggests that these outer membrane proteins could provide at least partial protec-
tion against heterologous challenge (23, 45).

The complexity of the leptospiral protein antigen profile indicates that additional protein antigens remain to be identi-
fied (39). To isolate leptospiral genes that express such anti-
gens, we screened a leptospiral genomic expression library with serum from a rabbit hyperimmunized with Leptospira kirsch-
neri serovar grippotyphosa. In this report, we describe initial characteriza-
tion of one of these proteins, a novel putative lipoprotein that we designated LipL45.

MATERIALS AND METHODS

Bacterial strains and media. L. kirschneri serovar grippotyphosa strain RM52 was isolated during an outbreak of porcine abortion in 1983 (53). Low- and high-passage L. kirschneri serovar grippotyphosa strain RM52 and other leptospiral strains were obtained from the National Leptospirosis Reference Center (National Animal Disease Center, Agricultural Research Service, U.S. Depart-
ment of Agriculture, Ames, Iowa) and were cultivated at 30°C in Johnson-Harris bovine serum albumin-Tween 80 medium (Bovuminar PLM-5 microbiological medium; Intergen) (31). Low-passage samples of the RM52 isolate were either stored in liquid nitrogen or passed in liquid media at 200 times to generate a high-passage form. The high-passage strain was unable to produce a lethal infection in hamsters at any dose and was able to infect hamsters only at a dose of ≥107 cells per animal by intraperitoneal inoculation. Low-passage forms of L. kirschneri serovar grippotyphosa strain ISU 82, Leptospira interrogans serovar canicola/portlandvere strains Mx 1 and CDC Nic 1808, and L. interro-
gans serovar pomona type kennevicki strain P10837-56 were generated by chal-
lenging 3-week-old Golden Syrian hamsters intraperitoneally with 5 × 107 cells of each organism. When clinical signs of illness developed or at least 14 days postinoculation, the hamsters were euthanized, and blood and kidney tissue were collected at necropsy and cultured in a semisolid medium which consisted of Bovuminar PLM-5 medium supplemented with 0.2% agar and 100 µg of 5-flu-
orouracil (Sigma) per ml. Experimental protocols involving rabbits and hamsters were approved by the VA Greater Los Angeles Healthcare System Animal Research Committee.

Expression and purification of recombinant LipL45. The portion of Sorf2 encoding the mature part of LipL45 was amplified with Pfbl Turbo DNA poly-
merase (Stratagene) by using the upstream primer Sorf2(21n)-1up (5'-TTGG ATCTCCTGAAGAGAACCTACCGGAAGTG-3') and the downstream primer Sorf2(17p)-2dn (5'-TCTCCGCAAGATCATCAGTTGCTGGCAGACCT-3'); the primers contained a BamHI site and a NotI site, respectively, near their 5' ends (underlined). After an initial 1 min of denaturation at 95°C, the reaction mixture was subjected to 15 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1.25 min. The final cycle was followed by an additional extension step consisting of 72°C for 2 min. The PCR product was purified using a Qiagen QIAquick gel purification kit and ligated into the BamHI site of pBluescript 12 or 15% poly-
acrylamide gel electro-
phoresis (PAGE) gels by using a discontinuous buffer system essentially as described previously (35).

Following electrophoresis, each gel was stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid-45% methanol) and transferred electrophoretically to a polyvinylidene difluoride membrane (Milli-

topore). The membranes were incubated with rabbit serum in blocking solution for 30 min (see below for the serum titers used), washed three times with PBS-T, incubated with donkey anti-rabbit immunoglobulin-gal linker (pCGGATTCCG), digested with a QAquick PCR purification kit, digested with EcoRI and BamHI, and separated by electrophoresis in a 0.8% preparative agarose gel. DNA that was 1 to 5 kb long was excised from the gel, purified with a QAquick gel extraction kit (Qiagen), ligated to λ TriExEAs by following the instructions provided by the supplier (Clontech), and packaged into λ heads with Gigapack III Gold packaging extract (Stratagene). The phage titer of the library was determined by infecting E. coli XLI-Blue. To screen the library, approximately 107 PFU was plated by using E. coli XLI-Blue, transferred to nitrocellulose membranes (Schleicher & Schuell) soaked with isopropanol-β-D-thiogalactopyranoside (IPTG), and processed as rec-

ommended by the manufacturer (Schleicher & Schuell). The membranes were then treated for 30 min with blocking solution consisting of 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T); then they were incubated for 2 h with rabbit antiserum to virulent L. kirschneri RM52 (27) diluted 1:1,000 in blocking solution, washed three times in PBS-T, and incubated for 30 min with donkey anti-rabbit immunoglobulin-gal linker (pCGGATTCCG) conjugate (Amersham) diluted 1:4,000 in blocking buffer, and finally washed three times in PBS-T. Lifts were developed with the ECL Western blot detection reagents (Amersham) and exposed to Hyperfilm (Amersham) to identify plaques that reacted with the rabbit antiserum. Each reactive plaque was stored at 4°C in 1 ml of SM (0.1 M NaCl, 0.8 mM MgSO4, 50 mM Tris-HCl [pH 7.5], 0.01% [wt/vol] gelatin) with 1 to 2 drops of chloroform. λ clones that reacted with the serum were purified further by two rounds of plaque purification. Finally, the plasmid embedded in the phage DNA was excised by infecting E. coli BM25.8 with the λ clones as described by the manufacturer of the expression vector (Clontech).

Gel electrophoresis and immunoblotting. Samples were solubilized in final sample buffer consisting of 0.5 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue in 20% glycerol. Proteins were separated in 12% PAGE gels precast Tri-glycine gels (BioWhitt-
taker Molecular Applications) or SDS–12 or 15% polyacrylamide gel electro-
phoresis (PAGE) gels by using a discontinuous buffer system essentially as described previously (27). Following electrophoresis, each gel was stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid-45% methanol) and transferred electrophoretically to a polyvinylidene difluoride membrane (Milli-

topore). The membranes were incubated with rabbit serum in blocking solution for 30 min (see below for the serum titers used), washed three times with PBS-T, incubated with donkey anti-rabbit immunoglobulin-gal linker (pCGGATTCCG) conjugate (Amersham) at a dilution of 1:5,000 in blocking solution for 30 min, and again washed three times with PBS-T. The membranes were developed with the ECL Western blot detection system (Amersham), and bands were visualized with Hyperfilm (Amersham).

DNA sequencing. The nucleotide sequences of the clones were assembled from individual sequences obtained by using a combination of primer walking and sequencing of nested deletions. Deletions were generated from plasmid clones by removing restriction fragments extending from inside an insert to the multicloning sites flanking the insert. Oligonucleotides were synthesized by Gibco BRL. The UCLA Core Sequencing Facility and the Yale/Keck Core DNA Sequencing Facility performed the sequencing reactions.

Expression and purification of recombinant LipL45. The portion of Sorf2 encoding the mature part of LipL45 was amplified with Pfbl Turbo DNA poly-
merase (Stratagene) by using the upstream primer Sorf2(21n)-1up (5'-TTGG ATCTCCTGAAGAGAACCTACCGGAAGTG-3') and the downstream primer Sorf2(17p)-2dn (5'-TCTCCGCAAGATCATCAGTTGCTGGCAGACCT-3'); the primers contained a BamHI site and a NotI site, respectively, near their 5' ends (underlined). After an initial 1 min of denaturation at 95°C, the reaction mixture was subjected to 15 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1.25 min. The final cycle was followed by an additional extension step consisting of 72°C for 2 min. The PCR product was purified using a Qiagen QIAquick gel purification kit and ligated into the BamHI site of pBluescript 12 or 15% polyacrylamide gel elec-

tronephoresis (PAGE) gels by using a discontinuous buffer system essentially as described previously (27).

Following electrophoresis, each gel was stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid-45% methanol) and transferred electrophoretically to a polyvinylidene difluoride membrane (Milli-

topore). The membranes were incubated with rabbit serum in blocking solution for 30 min (see below for the serum titers used), washed three times with PBS-T, incubated with donkey anti-rabbit immunoglobulin-
acrylamide (for His<sub>6</sub>-5ORF2 and His<sub>6</sub>-5ORFC2) and allowed to migrate into the separating gel during electrophoresis. A band containing a 250- to 500-kDa fusion protein was excised from the gel, desiccated, ground to a powder, dissolved in 1 ml of water, mixed with 1 ml of complete Freund’s adjuvant (Sigma), and inoculated subcutaneously and intramuscularly into New Zealand White rabbits (Harlan Sprague Dawley) that were free of leptosiral antibodies. Additional immunizations with similar amounts of fusion protein in powdered acrylamide gel mixed with incomplete Freund’s adjuvant (Sigma) were administered 4 and 8 weeks after the primary immunization. Blood was collected from the rabbits 10 weeks after the primary immunization and processed to obtain serum (28).

Antibody to GroEL from L. interrogans serovar copenhageni was a generous gift from B. Adler (Monash University, Clayton, Victoria, Australia) (1).

Southern blotting. Genomic DNA was extracted from L. kirschneri with a Wizard genomic DNA purification kit (Promega). A 2.5-kb portion of DNA was digested with 5 to 20 U of EcoRI or CIlI overnight in a 20-μl (final volume) mixture. DNA was fractionated using a 1% agarose gel and stained with ethidium bromide. A 1.0-kb portion was excised from the gel, desiccated, ground to a powder, dissolved in 1 ml of water, mixed with 1 ml of complete Freund’s adjuvant (Sigma), and inoculated subcutaneously and intramuscularly into New Zealand White rabbits (Harlan Sprague Dawley) that were free of leptosiral antibodies. Additional immunizations with similar amounts of fusion protein in powdered acrylamide gel mixed with incomplete Freund’s adjuvant (Sigma) were administered 4 and 8 weeks after the primary immunization. Blood was collected from the rabbits 10 weeks after the primary immunization and processed to obtain serum (28).

The reaction mixture (20 μl) was placed according to the instructions of the supplier of the Taq polymerase (Qiagen). The PCR mixture was subjected to initial denaturation step consisting of 95°C for 1 min and then 20 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 2 min and a final extension step consisting of 4 min at 72°C.

The membrane was subjected to prehybridization for 3 h at 42°C in hybridization solution containing 40% formamide, 4× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 10 mM Tris-HCl (pH 7.4), 10% dextran sulfate, 1× Denhardt’s solution, and 50 μg of salmon sperm DNA per ml in a hybridization tube in a rotisserie oven (Hybaid). Prior to hybridization, the biotin-labeled PCR probe was boiled for 10 min and snap cooled on ice. The denatured probe was mixed with hybridization buffer and incubated overnight with the membrane at 42°C. Following hybridization, the blot was washed twice (15 min each) in 2× SSC-0.1% SDS at room temperature (low stringency). A Phototop-Star chemiluminescent detection kit from New England Biolabs was used to visualize bands with Hyperfilm (Amersham).

To purify P31LipL45, by immunoprecipitation, 4× 10<sup>7</sup> leptospire cells were centrifuged, washed twice with 10 ml of PBS-5 mM MgCl<sub>2</sub>, resuspended in 5 ml of a solution containing 1% protein grade Triton X-100 (Calbiochem), 20 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, and mixed with a Nutator for 30 min at 4°C to solubilize the outer membrane. The Triton X-100-insoluble protoplasmic cylinder was pelleted by centrifugation for 15 min at 10,000 rpm in a Sorvall SM24 rotor. The supernatant fluid was mixed with 25 μl of LipL45 antiserum and 62.5 μl of a suspension containing 0.2 g of protein/ml on a Nutator for 30 min at 4°C. The mixture was centrifuged for 10 min at 3,500 rpm in the SM24 rotor to pellet the P31-antibody-Sephrose complex. The pellet was washed twice with 10 ml of Tris-HCl (pH 8)-0.01% Triton X-100 and resuspended in 50 μl of final sample buffer containing 25 mM phenylmethylsulfonyl fluoride. The proteins in the mixture were separated in an SDS-12%-PAGE gel and stained with Coomassie brilliant blue. Affinity-purified His<sub>6</sub>-LipL45 fusion protein was electrophoresed similarly in an SDS-PAGE gel and stained.

Both the 31-kDa band and the 46-kDa band, representing native P31LipL45 and recombinant His<sub>6</sub>-LipL45, respectively (see Fig. 6, lanes 1 and 2), were excised from the gel and soaked in 0.5 ml of water for 15 min at 4°C. Each gel slice was then cut into small pieces, which were soaked in 50 μl of V8 sample buffer (0.1 M Tris-HCl [pH 6.8], 0.2% SDS, 20% glycerol, 0.2% bromophenol blue) for 15 min at 4°C, heated for 10 min at 50°C, and placed in a well of an SDS-15% PAGE gel in a Hoefer SE 600 unit. A 25-μl portion of a solution containing 10 μg of V8 protease/ml in V8 sample buffer was added to the same well, and electrophoresis was immediately started. Electrophoresis was interrupted when the stack was 1 cm above the stacking gel-resolving gel interface to permit digestion of P31LipL45 by V8 protease. Electrophoresis was resumed 30 min later to separate the peptides in the resolving gel.

Cellular fractionation of Leptospira. The outer membrane of L. kirschneri was solubilized with Triton X-114 as described previously (24), except that 0.5% protease inhibitor cocktail (catalog no. P8849; Sigma) was included in the lysis buffer. The membrane was isolated and washed as described previously (44), with some minor modifications. Cells were suspended in lysis buffer (20 mM Tris-HCl [pH 8], 50 mM NaCl, 2 mM EDTA, 0.5% protease inhibitor cocktail). Then 2 mg of lysozyme was added, and the cell suspension was incubated on ice for 5 min. The cell suspension was frozen at −80°C and thawed three times with vigorous vortexing. The membrane was pelleted by centrifugation at 16,000×g for 30 min and resuspended in 600 μl of lysis buffer. Samples (100 μl) of the membrane suspension were mixed separately with 100 μl of 400 mM NaCl, 100 μl of 3.6 M urea, 100 μl of 1.2 M Na<sub>2</sub>CO<sub>3</sub>, and 100 μl of 1% Triton X-100 with the Nutator for 15 min. The membrane was recovered by centrifugation in a microcentrifuge for 15 min and resuspended in 166 μl of final sample buffer. Protein released into the supernatant fluid was precipitated with acetone and resuspended in 166 μl of final sample buffer. Each membrane pellet was resuspended in 166 μl of final sample buffer.

Immunohistochemistry. The methods used to perform an immunohistochemistry analysis and to obtain L. kirschneri-infected hamster kidney tissue have been described previously (4). Briefly, groups of three 5-week-old Golden Syrian hamsters (Harlan Sprague Dawley) were inoculated intraperitoneally with 10<sup>5</sup> virulent L. kirschneri RMC-2 cells. Hamsters that were still alive 28 days after this challenge were euthanized, and liver and kidney tissues were removed, fixed in formalin, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin stain or silver stain by the technique of Steiner and Steiner (50). Serial 5-μm sections of kidneys taken 28 days after infection with L. kirschneri were cut and placed on Probe-on Plus slides. Paraffin was removed from the sections with xylene and ethanol by standard procedures. The tissue sections were pretreated with 0.1% trypsin in 0.1 M Tris-HCl (pH 7.6)-0.1% CaCl<sub>2</sub> for 5 min at 37°C. Nonspecific staining of tissue sections was blocked by using 10% normal goat serum and incubation at room temperature for 20 min prior to overnight incubation at 4°C with primary antibody. LipL45 antiserum was used at a 1:6,000 dilution. The controls included kidney sections from uninfected hamsters treated with hyperimmune serum and from infected hamsters treated with normal rabbit serum or no primary antibody. Unbound primary antibody was removed, and the tissues were incubated at room temperature for 30 min with biotinylated goat anti-rabbit immunoglobulin (Vector). After washing, sections were incubated for 20 min at room temperature with a biotinylated secondary antibody (Vector). After washing, sections were incubated for 20 min at room temperature with a peroxidase-conjugated secondary antibody. The sections were counterstained with hematoxylin before dehydration in alcohols and Propar (a xylene substitute), and coverslips were mounted.

Nucleotide sequence accession number. The sequence of the 2.264-nucleotide insertion of clone 5, which encodes the lipL45 gene from L. kirschneri serovar grippotyphosa strain RM52, has been deposited in the GenBank database under accession number AF379683.

RESULTS

Isolation of the gene encoding LipL45. We constructed a leptospiral expression library by using genomic DNA from virulent L. kirschneri serovar grippotyphosa strain RM52. The library was screened with serum from a rabbit hyperimmunized with formalin-killed, virulent L. kirschneri, which resulted in isolation of 104 reactive λ bacteriophage clones. The plasmid DNA embedded in the λ bacteriophage sequence was excised from these clones and introduced into E. coli for immunoblot analysis. Figure 1 shows the reactivity of the serum used to screen the library with E. coli transformed with nine representative clones. The serum reacted with antigens ranging in size from 20 to 150 kDa and did not react with any protein expressed from plasmids excised from four nonreactive λ clones. Antigens ranging in size from 33 to 50 kDa are shown in the immunoblot in Fig. 1. Immunoblotting did not detect a signal with 28 clones, apparently because of the instability of the foreign protein expressed in E. coli or the small size of the antigen (data not shown).

To determine the identities of the clones, the 76 plasmid
clones expressing an antigen that was detected in the immuno
blot screening analysis were examined either by immunoblot analysis with sera specific for known leptospiral antigens (LipL32, LipL41, OmpL1, and GroEL) or by partial sequence analysis. Sequences were analyzed with BLASTX (http://www.
cbi.nlm.nih.gov/BLAST/). For clones harboring multiple open reading frames, a deletion analysis was performed to identify the gene expressing the antigen. Nine clones encoded one of the outer membrane lipoproteins (LipL32 or LipL41) (24, 45). Fifteen clones encoded the α, β, or β′ subunit of RNA polymerase. Of the 13 clones that encoded the β or β′ subunit (43), 11 expressed an antigen that was larger than 80 kDa. Based on this observation, many of the 14 unsequenced clones that expressed a high-molecular-mass antigen (>80 kDa) probably encoded the β or β′ subunit. In addition, three clones encoded dihydrolipoamide succinyltransferase, an enzyme involved in intermediary metabolism, and four clones encoded the heat shock protein GroEL (3). The sequences of four antigens expressed by nine clones did not significantly match any sequence in the databases (GenBank, EMBL). Two of the four novel antigens were predicted to have a cleavable signal sequence.

The remaining 22 of the 76 clones encoded one of two putative lipoproteins. LipL32, LipL41, OmpL1, and GroEL antisera did not react with any of these clones (data not shown). One of the 22 clones, clone 5, was selected for further analysis. The sequence of clone 5 revealed that there are two open reading frames harbored in a 2,264-bp insert (Fig. 2) (accession number AF379683). 5orf1 consists of the last 756 nucleotides of an open reading frame fused to vector sequences upstream of the insert, and 100 nucleotides downstream of 5orf1 is 5orf2, which is a 1,164-nucleotide open reading frame that is predicted to express a protein which is 388 amino acid residues long (Fig. 2). 5orf2 has two potential ATG start codons separated by three nucleotides. Because the upstream ATG is separated from the probable Shine-Dalgarno sequence by only two nucleotides (Fig. 2), translation is more likely to start from the downstream ATG. 5orf2 is followed by two consecutive stop codons. A potential E. coli σ70 promoter consisting of the sequences aTGGAtt and aacAAT in the −35 and −10 regions (with matches to consensus indicated by uppercase) separated by 16 nucleotides is upstream of 5orf2. Immediately downstream of 5orf2 is a sequence resembling a ρ-independent transcription terminator, an inverted repeat followed by several thymines. No sequence resembling a ρ-independent terminator was found between 5orf1 and 5orf2, raising the possibility that the genes are cotranscribed.

Deletion analysis of the insert harbored by clone 5 indicated that 5orf2 expresses the 45-kDa protein in E. coli (data not shown). The amino terminus of the protein encoded by 5orf2 appears to consist of a 21-amino-acid signal peptide sequence with a spirochetal lipobox containing the lipoprotein signal peptidease cleavage site Val→_Phe→_Asn→_Ala→_Cys→ (22). The processed protein is 367 amino acid residues long, and its calculated molecular weight is 39,813. A BLAST search revealed 98% amino acid sequence identity with the uncharacterized putative lipoprotein plp42 from L. interrogans (accession number AF320329). We designated the protein LipL45 to conform to the nomenclature used for other leptospiral lipoproteins (22).

**LipL45 levels are associated with culture passage.** To generate antibody against LipL45, the protein without its signal sequence was expressed with an amino-terminal His, tag in E. coli and purified by Ni²⁺-NTA affinity chromatography. A rabbit was immunized with the fusion protein to generate antibody against LipL45. An immunoblots of various isolates of L. kirschneri RM52 cultured from the blood or kidneys of two hamsters infected in the laboratory was probed with the LipL45 antisera (Fig. 3). These isolates were passaged serially in vitro less than five times. The LipL45 antisera detected a 45-kDa antigen in all of the low-passage isolates (Fig. 3, lanes 2 to 10). Hamster 1 isolates expressed more LipL45 than hamster 2 isolates, possibly because of outgrowth of different clonal populations that adapted to culture conditions at different rates. Interestingly, the 45-kDa band was not detected in the culture-attenuated form of L. kirschneri RM52 (Fig. 3, lane 1). The LipL45 antisera also reacted with a 31-kDa antigen (P31) expressed by both low- and high-passage strains.

The relationship of LipL45 detection with culture passage was examined in other low- and high-passage leptospiral strains. Nine virulent leptospiral strains representing L. kirschneri serovar grippotyphosa, L. interrogans serovar pomona, and L. interrogans serovar canicola were inoculated into hamsters for the purpose of culturing low-passage hamster isolates. Immunoblots of these low-passage strains demonstrated that four of the nine hamster isolates expressed full-length LipL45 (Fig. 4, lanes 1 to 5; data not shown). Expression of LipL45 was also examined in a variety of saprophytic and high-passage pathogenic strains of Leptospira and Leptonema representing most species and serogroups (Fig. 5). To control for loading, the blot was also probed with serum to Impl63, an inner membrane protein expressed by all pathogenic and saprophytic serovars of Leptospira examined to date. LipL45 was not detected in any of the strains, but all of the strains expressed the P31 antigen (Fig. 5). Interestingly, pathogenic serovars produced a P31 doublet, whereas saprophytic serovars produced a single band, a poten-
tially useful phenotypic marker for pathogenicity. The results of a short exposure that revealed the doublets are shown in Fig. 5. Longer exposures did not reveal full-length LipL45 (data not shown). These observations suggest that LipL45 levels depend in part on culture passage. Because the 45-kDa band was observed only in low-passage isolates and because low-passage Leptospira isolates are expected to be virulent, LipL45 is a virulence-associated protein.

LipL45 is converted into a 31-kDa antigen. To determine the relationship of P31 to LipL45, we first created His6 fusion proteins fused to the amino-terminal 96 amino acid residues of LipL45 (5ORF2N) and to the carboxy-terminal 88 amino acid residues of LipL45 (5ORF2C) (Fig. 2) and raised antiserum against each fusion protein. Immunoblot analysis showed that 5ORF2C antiserum reacted with both LipL45 and P31 (data not shown), demonstrating that P31 is antigenically related to the C-terminal 88 amino acid residues of LipL45. On the basis of these results, 5ORF2N antiserum would be expected to react with LipL45 and not with P31. However, we were not able to raise 5ORF2N antiserum; sera from two rabbits immunized with the N terminus of LipL45 did not react with either protein in immunoblots of low- and high-passage L. kirschneri isolates. Because the 5ORF2N fusion protein used for immunization had been solubilized in urea prior to purification, the leptospires were subjected to equivalent denaturing conditions by boiling the bacteria in final sample buffer containing urea.

FIG. 2. Sequence of the leptospiral gene encoding LipL45. The nucleotide sequence of clone 5 (accession number AF379683) from position 760 through position 2109 is shown. Probable \( /H11002 \)35 and \( /H11002 \)10 promoter sequences are indicated by large letters. The Shine-Dalgarno sequence is underlined. The spirochetal lipobox is indicated by boldface type, and the proposed signal peptidase II processing site is indicated by an arrow. The sequences of the PCR primers used to generate the probe for the Southern blot are indicated by multiple arrowheads. The putative \( /H9267 \)-independent transcriptional terminator is indicated by dashed arrows. The amino acid sequence used to generate serum to the C terminus of LipL45 (5ORF2C) is shaded.
prior to electrophoresis. Despite these precautions, the 5ORF2N antisera did not react with LipL45 or P31 (data not shown).

We wished to determine whether P31 was a fragment of LipL45 or a cross-reactive antigen expressed from a gene other than lipL45. While eight independent clones expressing LipL45 were isolated from our expression library, no clone expressing a 31-kDa or smaller protein with homology to LipL45 was isolated. This observation is consistent with P31 being a processing product of LipL45 rather than a 31-kDa protein expressed from a separate gene. Nevertheless, it is possible that antibodies that react with the 31-kDa protein were not present in the serum used to screen the expression library. Therefore,
we performed two experiments to examine this question more carefully.

First, the C termini of LipL45 and P31 were compared by proteolytic digestion. If the C terminus of LipL45 encompasses the 31-kDa protein, then serum raised against the C terminus of LipL45 should react with identical peptides generated by V8 protease digestion of LipL45 and P31. Gel-purified His$_6$-tagged LipL45 fusion protein and native P31 (see Materials and Methods) were used as substrates for proteolysis. The digestion products were subjected to immunoblot analysis with an anti-His$_6$ antibody (Fig. 6). Figure 6 shows that the digestion patterns for LipL45 (lane 4) and P31 (lane 3) were identical, indicating that P31 is derived from the C terminus of LipL45.

Second, a low-stringency Southern blot analysis in which low- and high-passage L. kirschneri genomic DNA were used was performed (Fig. 7). Each DNA was cut with EcoRI (Fig. 7, lanes 1 and 2) or ClaI (lanes 3 and 4); restriction sites for these enzymes are not found in the Sorf2 probe, which spans most of the reading frame (Fig. 2). As expected for a single-copy gene, both types of digestion resulted in a single band. The positions of molecular mass standards (in kilodaltons) are indicated on the right. The His$_6$-LipL45 protein migrated as a 46-kDa band (lane 1). Note that LipL45 was detected when a large number of high-passage leptospires were loaded onto the gel (lane 2).

Exploiting the unique properties of the nonionic detergent Triton X-114 (7), previous studies have shown that Triton X-114 solubilizes the outer membrane of L. kirschneri, leaving behind the protoplasmic cylinder consisting of the cytoplasm surrounded by the inner membrane (27, 56). As expected, these studies localized the penicillin-binding proteins to the protoplasmic cylinder of Leptospira (10, 27). Furthermore, when the temperature of the solubilized material is raised above the clouding point of the detergent to 37°C, LPS and outer membrane proteins LipL32, LipL36, LipL41, and OmpL1 partition into the detergent phase and periplasmic proteins partition into the aqueous phase (24, 25, 45). When Triton X-114 fractions were probed with LipL45 antiserum, P31$_{\text{LipL45}}$ was found to be distributed among the protoplasmic cylinder, the detergent phase, and the aqueous phase (Fig. 8A). One interpretation of these results is that P31$_{\text{LipL45}}$ is loosely bound to the inner membrane and/or the outer membrane. In contrast, outer membrane protein LipL36 was located entirely in the detergent phase, as described in a previous study (Fig. 8A) (25).

To investigate the relationship of LipL45 and P31$_{\text{LipL45}}$ with the lipid bilayer further, L. kirschneri was lysed with lysozyme, followed by several rounds of freezing and thawing. The membrane was pelleted by centrifugation and washed with various reagents (Fig. 8B) (44). Peripheral membrane proteins are solubilized by high salt concentrations, high urea concentrations, or high pH values, whereas integral membrane proteins are retained in the lipid bilayer following any of these treatments (20, 44). Both LipL45 and P31$_{\text{LipL45}}$ were found in the membrane pellet (Fig. 8B, lane 1). Neither protein could be removed with a high-salt wash (lanes 3 and 4). On the other hand, P31$_{\text{LipL45}}$ was washed off the membrane by urea, whereas LipL45 was resistant to urea treatment (lanes 5 to 8). Integral membrane protein OmpL1 could not be washed off the
membrane by any treatment (data not shown) (44). Lipoproteins LipL41 and LipL45 were released from the membrane by sodium carbonate treatment. Extremes of pH are known to hydrolyze the covalently attached lipid moieties that anchor lipoproteins to membranes (8). The results of the urea experiment suggest that P31LipL45 is a peripheral membrane protein loosely associated with the outer or inner membrane and can be dislodged under conditions that do not affect integral membrane proteins. LipL45 appears to be an integral membrane protein, presumably due to integration of its N-terminal lipid moiety. Apparently, Triton X-100 did not completely solubilize the membrane; LipL41 and P31LipL45 were partially removed from the membrane, and all LipL45 remained in the membrane pellet (Fig. 8B, lanes 10 and 11).

**FIG. 8.** Localization of LipL45 and P31LipL45. (A) High-passage *L. kirschneri* was fractionated with Triton X-114, and each fraction was subjected to immunoblot analysis with LipL45 (1:5,000) and LipL36 (1:4,000) antisera. Lane W, whole cells; lane P, insoluble pellet; lane A, aqueous fraction; lane D, detergent fraction. (B) Membrane fractions of virulent *L. kirschneri* were washed with buffer (lanes 2 and 3), NaCl (lanes 4 and 5), urea (lanes 6 and 7), Na₂CO₃ (lanes 8 and 9), or Triton X-100 (lanes 10 and 11) for 15 min. After this, the membrane was pelleted by centrifugation, and the pellets (P) and supernatant fluids (S) were subjected to immunoblot analysis with LipL45 and LipL41 antisera. Lane 1 contained unfractionated *L. kirschneri* (W).

**FIG. 9.** Variation of P31LipL45 levels with growth phase. (A) *L. interrogans* serovar pomona and *L. kirschneri* serovar grippotyphosa were inoculated into Bovuminar PLM-5 medium at a density of 5 × 10⁵ cells/ml, and the cultures were incubated at 30°C. The number of cells in each culture was determined by dark-field microscopy at different times. (B and C) Samples (1 × 10⁸ cells) obtained from the cultures at different times were examined by immunoblot analysis with LipL45 (1:4,000) and LipL41 (1:8,000) antisera. The strains used were *L. interrogans* serovar pomona strain P10637-46 (B) and *L. kirschneri* serovar grippotyphosa strain RM52 (C).

**P31LipL45 levels increase during the stationary phase.** In our studies of LipL45, we noticed variation in the levels of P31LipL45 after different cultures of *Leptospira* were harvested. We suspected that this variation resulted from changes in P31LipL45 expression with growth phase. To test this hypothesis, a small inoculum of *L. interrogans* serovar pomona was introduced into culture medium and allowed to grow to the stationary phase (Fig. 9A). Bacteria were harvested at daily intervals, and the level of P31LipL45 in each sample was determined by immunoblot analysis with the LipL45 antiserum (Fig. 9B). The immunoblot was simultaneously probed with the LipL41 antiserum; LipL41 levels are not affected by the growth phase of *Leptospira* (25). Equal loading was also confirmed by staining the total protein on the immunoblot with amido blue.
LipL45 is expressed by leptospires residing in the kidney. To examine LipL45 expression during infection, kidney sections of hamsters infected with virulent L. kirschneri were examined by immunohistochemical staining. We have used this technique to examine LipL32, LipL36, LipL41, and OmpL1 expression in infected hamsters in previous studies (4, 24). Kidney sections treated with preimmune rabbit antiserum showed no detectable reactivity (data not shown). In contrast, kidney sections treated with serum from an infected Golden Syrian hamster obtained 28 days after infection with virulent L. kirschneri was examined by performing an immunohistochemistry analysis with LipL45 antibody. The arrows indicate the reactive lumina of three kidney tubules. The lumen of one tubule (left) and one venule (top, containing a red blood cell) did not react with the LipL45 antibody.

FIG. 10. Immunohistochemistry of the kidney of an infected hamster. A kidney from an infected Golden Syrian hamster obtained 28 days after infection with virulent L. kirschneri was examined by performing an immunohistochemistry analysis with LipL45 antibody. The arrows indicate the reactive lumina of three kidney tubules. The lumen of one tubule (left) and one venule (top, containing a red blood cell) did not react with the LipL45 antibody.

**DISCUSSION**

We isolated a leptospiral gene encoding a novel protein designated LipL45. This protein is predicted to be a lipoprotein based on the sequence Val$_{\text{-4}}$-Phe$_{\text{-3}}$-Asn$_{\text{-2}}$-Ala$_{\text{-1}}$-Cys$_{\text{-1}}$ at the putative signal peptidase cleavage site found at the end of the 21-amino-acid signal peptide. The phenylalanine at the $-3$ position and the asparagine at the $-2$ position do not conform with the definition of the consensus lipobox sequence (Leu, Ala, Val)$_{\text{-4}}$-Leu$_{\text{-3}}$(Ala, Ser)$_{\text{-2}}$(Gly, Ala)$_{\text{-1}}$-Cys$_{\text{-1}}$, which is based on gram-negative lipoprotein signal peptidase cleavage sites (29). However, a recent alignment of spirochetal lipoproteins revealed a more relaxed consensus sequence (22). For example, several lipoproteins, including OspC from Borrelia burgdorferi and TpN29-35 from Treponema pallidum, have a phenylalanine residue at the $-3$ position. In addition, all members of the 2.9 lipoprotein family of B. burgdorferi have an asparagine residue at the $-2$ position (42). Although we have not determined whether LipL45 in virulent Leptospira isolates can be labeled by radiolabeled palmitate, the pH-sensitive association of LipL45 with the membrane and the inability of a high salt or urea concentration to remove LipL45 from the membrane are consistent with LipL45 being a lipoprotein (Fig. 8B).

LipL45 is predicted to be a 39.8-kDa protein after removal of its signal peptide. The discrepancy between the predicted molecular mass (39.8 kDa) and the apparent molecular mass (45 kDa) (Fig. 1) is similar to discrepancies observed with other spirochetal lipoproteins (24, 45, 52). Nevertheless, we cannot rule out the possibility that LipL45 is posttranslationally modified. Such modifications, if present, must be conserved in L. kirschneri and E. coli since LipL45 migrated as a 45-kDa protein when it was expressed in either organism. It is unlikely that the lipL45 gene that we have described is a product of ligation of two unrelated fragments of leptospiral DNA because the entire lipL45 gene was found in six independent clones.

Our initial characterization of LipL45 revealed several properties that are unique among known leptospiral proteins. LipL45 is a putative 45-kDa lipoprotein that is converted into a 31-kDa protein when it was expressed in either organism. It is unlikely that the lipL45 gene that we have described is a product of ligation of two unrelated fragments of leptospiral DNA because the entire lipL45 gene was found in six independent clones.
the actual mechanism of P31\textsubscript{LipL45} export, it is the first nonlipidated bacterial protein of which we are aware that exploits the lipoprotein secretion pathway to target itself to its final destination.

P31\textsubscript{LipL45} appeared as a doublet in all pathogenic \textit{Leptospira} species and as a single band in saprophytic \textit{Leptospira} species (Fig. 5). This observation indicates that P31\textsubscript{LipL45} is a marker for pathogenic \textit{Leptospira} isolates. We do not understand the molecular basis for this observation, but it may involve posttranslational modification or cleavage at multiple sites of LipL45 in the pathogens. The rate at which these events occur may be influenced by the growth rate, with the modification or cleavage event completed in the faster-growing saprophytic species.

P31\textsubscript{LipL45} is the first leptospiral peripheral membrane protein to be identified. Unlike integral membrane proteins, P31\textsubscript{LipL45} was washed off with urea, indicating that it is a peripheral membrane protein (Fig. 8B). P31\textsubscript{LipL45} was not washed off by high salt concentrations, indicating that electrostatic charge is not the primary mode of association of P31\textsubscript{LipL45} with the membrane. Our hypothesis is that following removal of the signal peptide and lipidation of the N-terminal cysteine, full-length LipL45 associates via its lipid moieties with the cytoplasmic membrane, with the protein extending into the periplasm. The protein is subsequently processed by an unknown protease, releasing P31\textsubscript{LipL45}, which seems to associate with both the inner and outer membranes of \textit{Leptospira} (Fig. 8). The only other spirochetal peripheral membrane proteins described to date are the GlpQ and TpLRR proteins of \textit{B. burgdorferi} and the P22-A antigen of \textit{B. burgdorferi} (46–48).

Another novel feature of P31\textsubscript{LipL45} is that its level changes with the growth phase of the organism. The P31\textsubscript{LipL45} level increases as a \textit{Leptospira} culture enters the stationary phase (Fig. 9). This effect may result either from increased transcriptional and/or translational synthesis of LipL45 or from a diminished rate of P31\textsubscript{LipL45} breakdown as \textit{Leptospira} enters the stationary phase. During infection of a mammalian host, virulent \textit{Leptospira} cells multiply rapidly in the blood before they are cleared by the reticuloendothelial system (17). Some of the leptospires that survive migrate to the proximal convoluted tubule of the kidney, where they are able to multiply to a high density (4, 16, 37). LipL45 was detected in infected hamster kidneys by immunohistochemical staining (Fig. 10). The microenvironment in the kidney tubules may simulate the stationary phase, causing leptospires to upregulate expression of certain proteins, including the P31\textsubscript{LipL45} form of LipL45. Expression of other leptospiral proteins may be downregulated while \textit{Leptospira} resides in the kidney. Indeed, LipL36 levels decline as \textit{Leptospira} enters the stationary phase and are not detected in leptospires colonizing the kidney (4, 25). We do not know whether the inducing signal for P31\textsubscript{LipL45} upregulation is created by nutrient depletion, cell density, or some other factor.

We found that detection of LipL45 is correlated with virulence (Fig. 3 to 5). We therefore identified LipL45 as a virulence-associated protein to distinguish it from true virulence factors. Construction of a virulent \textit{L. kirschneri} strain with a disrupted \textit{lipL45} gene will be necessary to determine whether LipL45 is a virulence factor (41). The \textit{lipL45} gene is the first leptospiral gene that expresses a virulence-associated protein to be isolated. A virulence-associated fibronectin-binding protein was recently identified as a band on a blot, but the observed molecular mass of this protein, 35 kDa, is too low for it to be LipL45 (38). LipL45 is a highly specific marker for virulence because it is not detected in culture-attenuated (high-passage) strains (Fig. 5), but it is a somewhat insensitive marker for virulence because of our failure to detect it in many low-passage (virulent) strains (Fig. 4; data not shown). The inability to detect LipL45 after culture passage of \textit{Leptospira} is probably due to increased expression or activity of the protease that cleaves LipL45 into P31\textsubscript{LipL45}. However, LipL45 is not completely suppressed in high-passage organisms; LipL45 was detected when large numbers of leptospires were probed in an immunoblot (Fig. 6, lane 2).

We have not eliminated the possibility that P31\textsubscript{LipL45} is a decay product rather than a processed product of LipL45. If it is a decay product, it is plausible that expression or activity of the protease or proteases that target LipL45 is suppressed in an infected host. A similar phenomenon has been described for the SpeB cytolethal protease in group A streptococcus. Examination of different clinical isolates of group A streptococcus demonstrated that SpeB expression is inversely correlated with the severity of disease (34). A potential substrate of the SpeB protease is the surface-exposed M protein, a virulence factor essential for group A streptococcus to evade complement-mediated lysis (34). Failure to downregulate SpeB expression or activity may result in inappropriate cleavage and inactivation of M protein during infection. Other bacterial pathogens are known to regulate protease expression in response to environmental cues (21).

The antigenicity of LipL45 allowed us to isolate the \textit{lipL45} gene from a leptospiral genomic expression library. A total of 40% of the clones isolated from the expression library harbored genes encoding lipoproteins, including LipL32 and LipL41. Previous studies with \textit{B. burgdorferi} and \textit{T. pallidum} have shown that lipoproteins are highly immunogenic when animals are immunized with whole bacteria (8, 13). Lipid moieties dramatically increase the antibody response to the peptide or protein to which they are attached (5, 15). Undoubtedly, other lipoprotein genes remain to be isolated from the leptospiral genome. At least eight proteins are labeled when \textit{Leptospira} is grown in the presence of radiolabeled palmitate (25). In addition, other leptospiral lipoproteins may be expressed only during infection, as demonstrated for several \textit{B. burgdorferi} lipoproteins (18, 51, 54). Surprisingly, up to 38% of the clones harbored genes for RNA polymerase subunits. It is possible that like GroEL, which represented 5% of the clones isolated from the expression library, the RNA polymerase subunits contain sequences that are highly stimulatory to the immune system (40).

Although the serum used to screen the library contained OmpL1 antibody (data not shown), we failed to clone the OmpL1 gene from the library, possibly because of its extreme lethality when it is expressed in \textit{E. coli}, even at low levels (26). Similarly, we were not able to isolate the gene encoding DnaK from the library; the immune system of the rabbit may not have recognized this ubiquitous protein because it could be a self-antigen. It is also possible that some genes encoding protein antigens were not isolated due to chance. In addition to the RNA polymerase subunits, we isolated two other genes that
are found in a wide range of bacteria and encode two enzyme subunits involved in metabolism. The isolation of genes encoding cytoplasmic proteins indicates that whole-cell immunization results in production of antibody against antigens that may not be accessible to the host immune system during infection by live *Leptospira*. Indeed, serum from infected hamsters did not react with some antigens recognized by the rabbit serum used to screen the library (data not shown).

In conclusion, we isolated a gene encoding a virulence-associated lipoprotein, LipL45. Our results suggest that LipL45 is an inner membrane protein whose C terminus is released as a 31-kDa peripheral membrane protein. P31LipL45 is associated with both the inner and outer membranes. The amount of P31LipL45 in leptospires increases dramatically during the stationary phase, indicating that expression is environmentally regulated. Immunohistochemical and serologic studies with the hamster model of leptospirosis indicated that LipL45 or P31LipL45 or both are expressed during infection. The level of expression appears to be relatively low, since the immunohistochemical reaction is less intense that the reactions seen with other leptospiral antigens (Fig. 10) and few sera from human leptospirosis patients react with recombinant LipL45, as determined by enzyme-linked immunosorbert assays (4, 19, 24). An important survival strategy of pathogenic bacteria is to restrict expression of key immunoprotective antigens during infection of their mammalian hosts. Further studies are under way to determine whether LipL45 or its processed form, P31LipL45, is a target of a protective immune response.

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