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LipL21 Is a Novel Surface-Exposed Lipoprotein of Pathogenic *Leptospira* Species

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Leptospira **is the etiologic agent of leptospirosis, a bacterial zoonosis distributed worldwide. Leptospiral lipopolysaccharide is a protective immunogen, but the extensive serological diversity of leptospires has inspired a search for conserved outer membrane proteins (OMPs) that may stimulate heterologous immunity. Previously, a global analysis of leptospiral OMPs (P. A. Cullen, S. J. Cordwell, D. M. Bulach, D. A. Haake, and B. Adler, Infect. Immun. 70:2311-2318, 2002) identified pL21, a novel 21-kDa protein that is the second most abundant constituent of the *Leptospira interrogans* serovar Lai outer membrane proteome. In this study, we identified the gene encoding pL21 and found it to encode a putative lipoprotein; accordingly, the protein was renamed LipL21. Southern hybridization analysis revealed the presence of *lipL21* in all of the pathogenic species but in none of the saprophytic species examined. Alignment of the LipL21 sequence from six strains of *Leptospira* revealed 96 to 100% identity. When specific polyclonal antisera to recombinant LipL21 were used, LipL21 was isolated together with other known leptospiral OMPs by both Triton X-114 extraction and sucrose density gradient membrane fractionation. All nine strains of pathogenic leptospires investigated by Western blotting, whether culture attenuated or virulent, were found to express LipL21. In contrast, the expression of LipL21 or an antigenically related protein could not be detected in nonpathogenic *L. biflexa*. Infected hamster sera and two of eight human leptospirosis sera tested were found to react with recombinant LipL21. Native LipL21 was found to incorporate tritiated palmitic acid, consistent with the prediction of a lipoprotein signal peptidase cleavage site. Biotinylation of the leptospiral surface resulted in selective labeling of LipL21 and the previously known OMPs LipL32 and LipL41. These findings show that LipL21 is a surface-exposed, abundant outer membrane lipoprotein that is expressed during infection and conserved among pathogenic *Leptospira* species.**

Leptospira is a genus of spirochetal bacteria and the causative agent of leptospirosis, a zoonotic disease with a global distribution. An international survey of human leptospirosis deduced that approximately 100,000 severe cases (requiring hospitalization) occur annually (42). This figure is likely to be a significant underestimate due to the lack of diagnostic and epidemiologic resources in many areas of endemicity.

The distribution of the approximately 230 recognized serovars of pathogenic leptospires may be restricted geographically (13). The extensive serovar diversity has been attributed to differences in the structure and composition of lipopolysaccharide (LPS) (6). Much work has focused on the role of leptospiral LPS in immunity (14, 29), and the genetics of LPS biosynthesis have been partly elucidated (6, 7, 10, 11). Preparations of leptospiral LPS can elicit protective immunity, but this immunity is generally serovar specific (13). Consequently, the focus of research on protective antigens has shifted toward conserved outer membrane proteins (OMPs), which may be able to stimulate heterologous immunity.

Three classes of leptospiral OMPs have been identified. The most abundant class comprises the outer membrane lipoproteins and includes the major OMP and immunodominant protein antigen LipL32 (15, 18), the in vivo-down-regulated protein LipL36 (4, 19), LipL48 (20), and the surface-exposed protein LipL41 (39). Consistent with the theory of paucity of transmembrane proteins postulated for *Treponema pallidum* (34) and despite much investigation, only one rare transmembrane OMP has been identified. This protein has been designated OmpL1 and is thought to function as a heat-modifiable porin (17, 38). The third class, comprising the peripheral membrane proteins, contains one member, $P31_{LipL45}$, which exploits the lipoprotein secretion pathway to target itself to both the inner and the outer membranes (32).

Most of the genes encoding leptospiral OMPs have been identified in independent studies by probing of gene libraries with degenerate oligonucleotide probes or hyperimmunized rabbit sera. Recently, a more systematic approach was used to identify novel leptospiral OMPs (8). In that study, outer membrane preparations from leptospires grown under a variety of environmental conditions were separated by two-dimensional gel electrophoresis and the resolved proteins were analyzed by mass spectrometry. However, the usefulness of this approach is limited by the availability of a genome sequence against which to compare the mass spectrometry data. Currently, there are at least four leptospiral genome sequencing projects in the final

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TABLE 1. Oligonucleotides used in this study

Oligonucletide	Sequence	Target
PC1	5′-TCC AGT ACT GAC ACA GGA C-3′	5′ Primer used to amplify *lipL21* for sequencing; used in combination with PC2 or PC4
PC2	5′-TTG TTT GGA AAC CTC TTG A-3′	3′ Primer used to amplify *lipL21* for sequencing; used in combination with PC1 or PC3
PC3	5′-ACT CGG TAA AGT ATT AGC-3′	5′ Primer used to amplify *lipL21* for sequencing; used in combination with PC2 or PC4
PC4	5′-GGG TTT GTA AAT ATA TCG-3′	3′ Primer used to amplify *lipL21* for sequencing; used in combination with PC1 or PC3
PC5	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAG TAC TGA CAC AGG ACA-3′	5′ Primer used to amplify *lipL21* for λ site-specific recombination into pDONR201; incorporates a modified *attB* site
PC6	5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTT GGA AAC CTC TTG AG-3′	3′ Primer used to amplify *lipL21* for λ site-specific recombination into pDONR201; incorporates a modified *attB* site.
PC7	5′-TCG CGT TAA CGC TAG CAT GGA TCT C-3′	5′ *attL* sequencing primer; used to sequence the insert of the pDONR201 construct after site-specific recombination
PC8	5′-GTA ACA TCA GAG ATT TTG AGA CAC-3′	3′ *attL* sequencing primer; used to sequence the insert of the pDONR201 construct after site-specific recombination

stages of sequence annotation or publication. Analysis of the ensuing genome data will provide a subset of putative membrane proteins from which a comprehensive set of OMPs may be determined experimentally.

Immunoprotection studies with recombinant antigens in animal models of leptospirosis have met with only partial success (5, 21). Thus, the identification of additional OMPs, particularly those that are surface exposed, is of prime importance for the development of recombinant subunit vaccines that will elicit heterologous immunity.

In this study, the gene encoding the second most abundant protein in the *Leptospira interrogans* serovar Lai outer membrane proteome was identified and designated *lipL21*. The LipL21 sequence is well conserved among pathogenic leptospires but could not be detected in saprophytic strains. LipL21 is surface exposed and immunogenic, being recognized by immune sera from humans and hamsters infected with *Leptospira*.

MATERIALS AND METHODS

Bacterial strains and media. The following strains of *Leptospira* were grown at 30°C in EMJH (26) or bovine serum albumin-Tween 80 medium (Bovuminar PLM-5 microbiological medium; Intergen): *L. biflexa* serovar Patoc strain Patoc I, *L. borgpetersenii* serovar Hardjo strains HB-15B/93U and L550, *L. inadai* serovar Lyme strain 10, *L. interrogans* serovar Bratislava strain AS-05, *L. interrogans* serovar Canicola strain CA29, *L. interrogans* serovar Copenhageni Li-133, *L. interrogans* serovar Lai strain Lai, *L. interrogans* serovar Pomona strains PO-01 and RZ11, *L. kirschneri* serovar Grippotyphosa strain RM52, *L. kirschneri* serovar Mozdok strain 5621, *L. noguchii* serovar Fort Bragg strain Fort Bragg, *L. noguchii* serovar Proechymis strain LT796, *L. santarosai* serovar Bakeri strain LT79, *L. wolbachii* serovar Biflexa strain Codice, and *L. weilii* serovar Celledoni strain Celledoni. *Escherichia coli* DH5α was used for cloning and was cultured in Luria-Bertani (LB) broth or on 1.5% LB agar at 37°C overnight. *E. coli* BL21-SI (Invitrogen) was used for protein expression and was cultured at 37°C in LB medium without NaCl but containing the appropriate antibiotics.

Oligonucleotides. Oligonucleotides used in this study were synthesized by GIBCO BRL and are listed in Table 1.

DNA manipulations. Chromosomal DNA from *Leptospira* was prepared by using the cetyltrimethylammonium bromide precipitation method (3), while plasmid DNA was isolated by using a QIAprep spin miniprep kit (Qiagen). The *lipL21* sequence was amplified by high-fidelity PCR for double-stranded DNA sequencing with an Expand high-fidelity PCR kit (Roche). DNA sequencing was performed at the Yale/Keck Core DNA Sequencing Facility. Standard methods for molecular biology were performed essentially as described by Sambrook et al. (36).

DNA and protein sequence analyses. Sequence data were analyzed by using Sequencher 3.1 (GeneCodes), while DNA and protein database comparisons were made by using the BLAST programs of Altschul et al. (1, 2). Multiple sequence alignments and hydrophophilicity analysis were performed by using the Lasergene (DNAstar) software package.

Southern hybridization. After electrophoresis, *Eco*RI-digested genomic DNA was transferred to a positively charged nylon membrane (Roche) by capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0). Overnight hybridization and subsequent washings were performed under high-stringency conditions at 68°C as recommended in the Roche digoxigenin labeling and detection kit instructions. The *lipL21* probe was labeled by PCR amplification in the presence of digoxigenin DNA labeling mix (Roche) as specified by the manufacturer.

Gel electrophoresis and immunoblotting. Samples were solubilized in a final sample buffer consisting of 50 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% PAGEr Gold precast Tris-glycine gels (BioWhittaker Molecular Applications) or SDS–12 or 15% polyacrylamide gels by using a discontinuous buffer system essentially as described previously (30). After electrophoresis, each gel was stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid–45% methanol) or transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). The membranes were incubated with rabbit (diluted 1/5,000 to 1/20,000), hamster (diluted 1/2,000), or human (diluted 1/1,000) serum in blocking solution (5% skim milk powder and 0.05% Tween 20 in phosphate-buffered saline [PBS]); washed three times with PBS containing 0.1% Tween 20 (PBS-T); for 30 min; incubated with donkey anti-rabbit (Amersham), goat anti-hamster (Jackson ImmunoResearch Laboratories), or sheep anti-human (Silenus) immunoglobulin-horseradish peroxidase conjugates 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 an enhanced chemiluminescence Western blot detection system (Amersham), and bands were visualized with Hyperfilm (Amersham).

Recombinant protein expression and purification. PCR was used to amplify *lipL21* from *L. kirschneri* serovar Grippotyphosa strain RM52 with primers designed to engineer *attB* sites into the final product (Table 1). BP clonase (Invitrogen) was used to mediate λ site-specific recombination between the *attB*-flanked PCR product and the *attL*-containing plasmid pDONR201 (Invitrogen). *lipL21* was then transferred from the entry vector pDONR201 to the protein expression vector pDEST17 by using LR clonase (Invitrogen). Plasmid DNA was isolated from the *lipL21* pDEST17 clone and used to transform the expression strain *E. coli* BL21-SI. Cultures of the expression strain were grown to an absorbance at 600 nm of 0.6 and induced for 3 h with 0.3 M NaCl. The bacteria were lysed by sonication, and inclusion bodies containing the fusion protein were solubilized in 8 M urea–10 mM Tris-HCl–100 mM NaH_2PO_4 (pH 8.0). An

N-terminal hexahistidine-tagged fusion protein was subsequently purified by using an Ni^{2+}-nitrilotriacetic acid affinity column according to the manufacturer's instructions (Qiagen). LipL32 and LipL36 were prepared as described previously (18, 19).

Production of polyclonal antisera. Purified hexahistidine-tagged LipL21 was loaded onto a preparative SDS–12% polyacrylamide gel and allowed to migrate into the separating gel during electrophoresis. A band containing 1.5 mg of fusion protein was excised from the gel, divided into six equal pieces, and desiccated. Dessiccated gel pieces corresponding to 250 μg of recombinant protein were 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 leptospiral antibodies. Additional immunizations with similar amounts of fusion protein in powdered acrylamide gel mixed with incomplete Freund's adjuvant (Sigma) were administered 2 and 4 weeks after the primary immunization. Serum was obtained from the rabbits 5 weeks after the primary immunization (23). Antisera to LipL31 (20), LipL36 (19), LipL41 (39), and ImpL63 (20) were prepared as described previously.

Hamster infection. Sera were collected from hamsters infected with culture-derived or host-derived *L. kirschneri* serovar Grippotyphosa strain RM52 as described previously (4). Briefly, 5-week-old golden Syrian Hamsters (Harlan Sprague-Dawley) were inoculated intraperitoneally with 10^5 or 10^6 *L. kirschneri* organisms from a liquid culture (culture derived) or with leptospires recovered from the liver tissue of moribund weanling hamsters (host derived).

MAT. The microscopic agglutination test (MAT) (12) was perfomed by using viable serovars Australis, Canicola, Copenhageni, Grippotyphosa, Hardjo, Pomona, Tarrasovi, and Zanoni as antigens and sera at final dilutions (including antigens) of 1/50 to 1/32,000.

Triton X-114 extraction. *L. kirschneri* serovar Grippotyphosa strain RM52 outer membrane material was extracted by using a previously described method (18). Briefly, leptospires were washed in PBS–5 mM $MgCl_2$ and then extracted in the presence of 0.5% protein-grade Triton X-114 (Calbiochem)–150 mM NaCl–10 mM Tris (pH 8)–1 mM EDTA at 4°C. The insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. After centrifugation, 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 10 min at 1,000 × *g*. The detergent and aqueous phases were then separated and precipitated with acetone.

Alkaline plasmolysis and membrane fractionation. Membrane vesicles were prepared as described previously (20). Briefly, leptospires were washed, resuspended in 20 mM Tris (pH 9)–1 M NaCl–27% (wt/vol) sucrose–2 mM EDTA buffer, and subjected to high-speed vortexing with a microstir bar. After treatment with DNase and RNase and removal of the insoluble material by centrifugation, the supernatent was loaded onto a 27 to 55% (wt/vol) sucrose gradient. Appropriate fractions were then collected for subsequent analyses.

Radioimmunoprecipitation and fluorography. A total of 200 μCi of [^{3}H]palmitic acid (Amersham) was added to 25 ml of *L. kirschneri* serovar Grippotyphosa strain RM52 culture at a cell density of 10^7 cells/ml. When a cell density of 10^9 cells/ml was obtained, the cells were harvested by centrifugation. A total of 10^{10} cells were resuspended in boiling buffer (2% SDS, 50 mM Tris-Cl [pH 8.0]), boiled for 10 min, and lysed by sonication. Insoluble material was removed by centrifugation at 12,000 × *g*. Native LipL21 was immunoprecipitated from this mixture by the addition of 0.2 ml of LipL21 antiserum and 0.2 ml of protein A slurry, incubation at 4°C for 30 min, and centrifugation at 12,000 × *g*. The pellet was washed three times in radioimmunoprecipitation buffer (1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-Cl [pH 8.0]) before being resuspended in final sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). The sample was separated by SDS-PAGE, and the gel was treated with Amplify solution (Amersham). An image was obtained by incubating the dried gel in a film cassette with preflashed Hyperfilm-MP (Amersham) at −70°C for 1 week.

Surface biotinylation. *L. kirschneri* serovar Grippotyphosa strain RM52 was grown to mid-log phase, washed twice in PBS (pH 8.0), and resuspended in PBS containing 0.5 mg of sulfosuccinimidyl-6-(bitinamido)hexanoate (Sulfo-NHS-LC-Biotin) (Pierce)/ml. The labeling reaction was allowed to proceed for 1 min before the residual Sulfo-NHS-LC-Biotin was quenched by the addition of Tris (pH 8.0) to a final concentration of 50 mM. Inactivated Sulfo-NHS-LC-Biotin was removed by two washes with PBS (pH 8.0). LipL21 was immunoprecipitated from a lysate of 10^9 surface biotinylated cells as described above. Lysates of 10^9 surface biotinylated cells or immunoprecipitated LipL21 were resolved by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was blocked overnight in 5% (wt/vol) skim milk buffer, washed twice in PBS-T, and incubated with streptavidin-horseradish peroxidase conjugate (Amersham) at a dilution of 1/20,000 in PBS-T for 30 min. The membrane was washed six times with PBS-T and developed with the enhanced chemiluminescence Western blot detection system before visualization with Hyperfilm (Amersham).

Nucleotide sequence accession numbers. The nucleotide sequences of *lipL21* from *L. interrogans* serovar Lai, *L. interrogans* serovar Pomona, *L. interrogans* serovar Bratislava, *L. kirschneri* serovar Grippotyphosa, *L. kirschneri* serovar Mozdok, and *L. borgpetersenii* serovar Hardjo have been deposited in GenBank under the accession numbers AY187271, AY187273, AY187269, AY187270, AY187272, and AY187274, respectively.

RESULTS

Identification and distribution of *lipL21*. In a previous study, Cullen et al. obtained the peptide sequence from pL21, the second most abundant constituent of the *L. interrogans* serovar Lai outer membrane, by electrospray-ionization tandem mass spectrometry (8). This peptide sequence was used to search the in-progress genome sequence of *L. borgpetersenii* serovar Hardjo. A match to a translated open reading frame of 564 bp, which encoded a protein of 188 amino acids with a predicted molecular mass of 19.8 kDa, was identified (GenBank accession number AY187274). At the time of the initial identification (8), no proteins with significant similarity were identified in the public databases. Subsequently, a homologous sequence was identified in the *L. interrogans* serovar Lai genome sequence (GenBank accession number NP_710192). The pL21 amino acid sequence was found to contain a 17-amino-acid signal peptide and a putative lipoprotein signal peptidase (LSP) cleavage site ($F_{-3}A_{-2}A_{-1}C_{+1}$) (16, 41). In accordance with standard nomenclature for leptospiral lipoproteins, the protein was renamed LipL21.

A 400-bp *lipL21* probe was synthesized and used to detect the presence of similar DNA sequences in a panel of pathogenic and environmental leptospires by high-stringency Southern hybridization (data not shown). A single copy of the *lipL21* gene was present in all five pathogenic *Leptospira* species analyzed (*L. interrogans* serovar Lai, *L. interrogans* serovar Pomona, *L. kirschneri* serovar Grippotyphosa, *L. kirschneri* serovar Mozdok, *L. borgpetersenii* serovar Hardjo, *L. santarosai* serovar Bakeri, and *L. noguchii* serovar Proechymis). However, no hybridizing bands were detected when genomic DNAs from the environmental leptospires *L. biflexa* serovar Patoc and *L. wolbachii* serovar Biflexa were probed. Oligonucleotide primers flanking the sequence were designed to amplify the *lipL21* open reading frame from five other strains of pathogenic *Leptospira* for DNA sequencing. The translated *lipL21* sequences from *L. interrogans* serovar Lai, *L. interrogans* serovar Pomona, *L. interrogans* serovar Bratislava, *L. kirschneri* serovar Grippotyphosa, *L. kirschneri* serovar Mozdok, and *L. borgpetersenii* serovar Hardjo were found to share 96 to 100% identity (GenBank accession numbers AY187271, AY187273, AY187269, AY187270, AY187272, and AY187274, respectively).

The sequence encoding the mature protein (without the hydrophobic signal peptide) was cloned into the protein expression vector pDEST17. Introduction of the construct into BL21-SI cells resulted in high-level expression of the N-terminal hexahistidine-tagged fusion protein. Recombinant LipL21 was purified by immobilized metal affinity chromatography and used to raise polyclonal antiserum in New Zealand White rabbits. To assess the level and distribution of LipL21 expression, immunoblot analysis was performed on a panel of *Lep-*

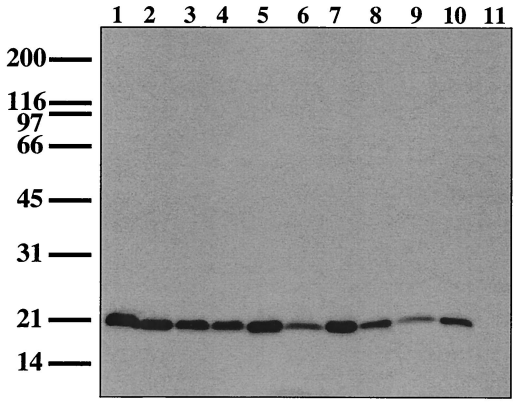

FIG. 1. Western blot of a panel of *Leptospira* species obtained by using LipL21 antiserum. Lanes contained whole-cell lysates of virulent isolates of *L. kirschneri* serovar Grippotyposa (lane 1), *L. interrogans* serovar Canicola (lane 2), and *L. interrogans* serovar Copenhageni (lane 3) and whole-cell lysates of culture-attenuated *L. interrogans* serovar Pomona (lane 4), *L. kirschneri* serovar Grippotyphosa (lane 5), *L. noguchii* serovar Fort Bragg (lane 6), *L. borgpetersenii* serovar Hardjo (lane 7), *L. weilii* serovar Celledoni (lane 8), *L. santarosai* serovar Bakeri (lane 9), *L. inadai* serovar Lyme (lane 10), and *L. biflexa* serovar Patoc (lane 11). LipL21 antiserum detected a single band of 21 kDa in each of the lanes containing a pathogenic *Leptospira* species. The positions of standard molecular mass markers (in kilodaltons) are indicated on the left.

tospira species with LipL21 antiserum (Fig. 1), which reacted with a single band of 21 kDa. Three virulent low-passage isolates of *Leptospira* were included in the panel (Fig. 1, lanes 1 to 3) to assess whether there were variations in the levels of expression between virulent and culture-attenuated organisms. LipL21 could be detected in all seven species of pathogenic leptospires examined, although the levels of expression in serovars Bakeri and Fort Bragg were slightly reduced (Fig. 1, lanes 6 and 9, respectively). No significant difference in the levels of LipL21 expression was observed between virulent and culture-attenuated leptospires. No LipL21 was detected in nonpathogenic *L. biflexa* serovar Patoc.

Cellular localization of LipL21. Two independent methods of cellular fractionation were used to confirm the cellular location of LipL21. Due to the association of peptidoglycan with the cytoplasmic membrane rather than the outer membrane in spirochetes (25, 28), the outer membrane can be solubilized with low concentrations of Triton X-114 (0.1 to 1%) without disruption of the cytoplasmic membrane. Subsequently, the contaminating hydrophilic periplasmic proteins can be removed by temperature-induced phase separation with TX-114. Recently, an alternative method for isolating leptospiral outer membranes was developed (20). This method involves the generation of membrane vesicles by alkaline plasmolysis and their subsequent separation by sucrose density gradient ultracentrifugation. Western blotting of these membrane vesicle and TX-114 fractions with antisera to several proteins with known cellular locations as well as LipL21 was performed. Antisera to the inner membrane proteins LipL31 and ImpL63 were included to show that the outer membrane fractions were free from contamination with inner membrane material. Antisera to two previously characterized leptospiral OMPs that differ in their properties during TX-114 extraction were included for comparison (Fig. 2). Both the TX-114 detergent phase and the outer membrane vesicle fractions contained the OMPs LipL36 and LipL41 and the novel protein LipL21. Notably, the inner membrane markers LipL31 and ImpL63 were absent from the TX-114 detergent phase and the outer membrane vesicle fractions. Unlike that of LipL36, solubilization of LipL21 by TX-114 was incomplete, as some material remained in the protoplasmic cylinder fraction, a behavior also exhibited by the leptospiral OMPs LipL41 (39) and OmpL1 (17).

Immunological reactivity of LipL21 with hamster and human sera. To determine whether LipL21 is expressed during infection and is immunogenic, sera were collected from four hamsters inoculated with leptospires from infected hamster

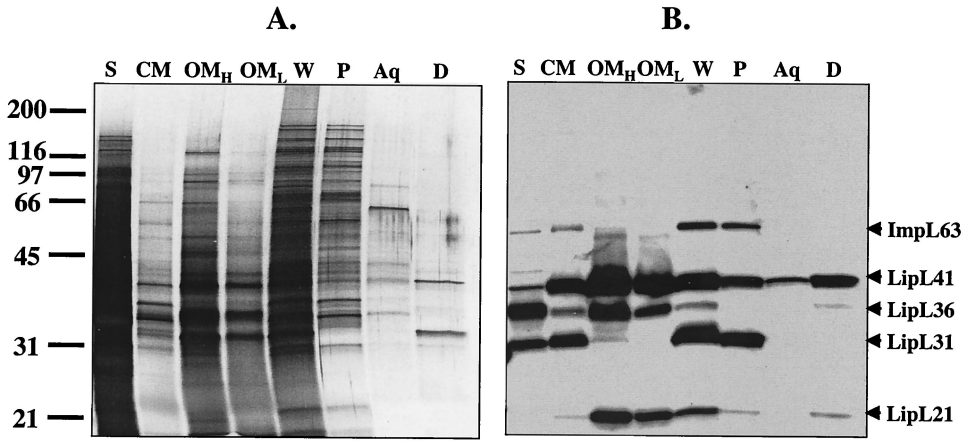

FIG. 2. Behavior of LipL21 and other leptospiral proteins during membrane fractionation by two independent methods. (A) Silver-stained SDS-polyacrylamide gel of soluble material from the top of the sucrose gradient (S), cytoplasmic membrane sucrose gradient fraction (CM), heavy outer membrane sucrose gradient fraction (OM_H), light outer membrane sucrose gradient fraction (OM_L), whole *L. kirschneri* cells (W), protoplasmic cylinders from TX-114 extraction (P), aqueous-phase fraction from TX-114 extraction (Aq), and detergent-phase fraction from TX-114 extraction (D). (B) Western blot of SDS-PAGE-separated sucrose gradient and TX-114 fractions (lanes as described above) with ImpL63, LipL41, LipL36, LipL31, and LipL21 antisera. The identities of individual proteins are indicated on the right, and the positions of standard molecular mass markers (in kilodaltons) are indicated on the left.

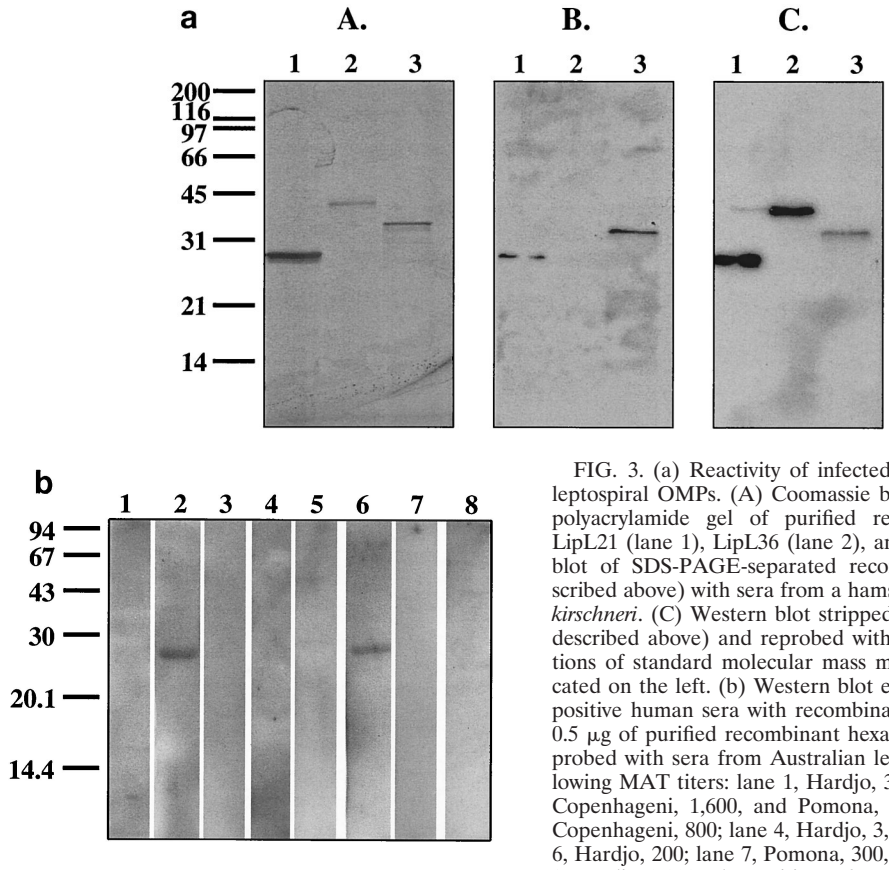

FIG. 3. (a) Reactivity of infected hamster sera with recombinant leptospiral OMPs. (A) Coomassie brilliant blue R-250-stained SDS-polyacrylamide gel of purified recombinant hexahistidine-tagged LipL21 (lane 1), LipL36 (lane 2), and LipL32 (lane 3). (B) Western blot of SDS-PAGE-separated recombinant proteins (lanes as described above) with sera from a hamster infected with host-derived *L. kirschneri*. (C) Western blot stripped of hamster antibodies (lanes as described above) and reprobed with penta-His antiserum. The positions of standard molecular mass markers (in kilodaltons) are indicated on the left. (b) Western blot examining the reactivity of MAT-positive human sera with recombinant LipL21. Each strip contained 0.5 μg of purified recombinant hexahistidine-tagged LipL21 and was probed with sera from Australian leptospirosis patients with the following MAT titers: lane 1, Hardjo, 3,200, and Tarrasovi, 800; lane 2, Copenhageni, 1,600, and Pomona, 800; lane 3, Canicola, 200, and Copenhageni, 800; lane 4, Hardjo, 3,200; lane 5, Australis, 1,600; lane 6, Hardjo, 200; lane 7, Pomona, 300, and Copenhageni, 200; and lane 8, Hardjo, 3,200. The positions of standard molecular mass markers (in kilodaltons) are indicated on the left.

liver, three hamsters challenged with virulent *L. kirschneri* RM52, and one uninfected hamster. In Western blotting experiments, all seven infected hamster sera recognized recombinant LipL21, while the uninfected control sera showed no reaction. Controls included recombinant forms of immunodominant protein antigen LipL32 and in vivo-down-regulated OMP LipL36 (Fig. 3a). Hamster sera recognized recombinant LipL21 and LipL32 but not LipL36. When the blotting membrane was stripped of antibody and reprobed with pentahistidine antibody, all of the recombinant proteins could be detected. Eight MAT-positive human sera were selected randomly from a bank of sera from Australian leptospirosis patients. In Western blotting experiments with this panel of sera, two of the eight sera recognized recombinant LipL21 (MAT titers: Copenhageni, 1,600; Pomona, 800; and Hardjo, 200) (Fig. 3b). No reactivity with LipL21 was observed when sera from eight nonleptospirosis patients were similarly examined (data not shown).

Lipid modification of LipL21. Analysis of the amino acid sequence of the protein encoded by *lipL21* revealed a putative LSP recognition sequence. Leptospires grown in the presence of [^{3}H]palmitic acid and separated by SDS-PAGE were shown to selectively incorporate the radiolabeled fatty acid into proteins of 21, 24, 32, 41, 45, 60, 62, 86, and 96 kDa (Fig. 4). The four most prominent bands appeared at 21, 25, 32, and 41 kDa, excluding the diffuse material below 14 kDa that may represent incorporation of the labeled fatty acid into LPS. When LipL21 was immunoprecipitated from leptospires grown in the presence of [^{3}H]palmitic acid and separated by SDS-PAGE, it was shown to incorporate the labeled fatty acid and to resolve at a mass similar to that of the prominent 21-kDa band from the whole-cell material.

Surface exposure of LipL21. To determine whether LipL21 was exposed on the leptospiral cell surface, intact cells were surface labeled in the absence of organic solvents with the

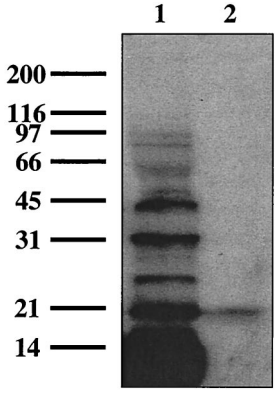

FIG. 4. Autoradiograph of whole-cell lysate of *L. kirschneri* grown in the presence of [^{3}H]palmitic acid (lane 1) and of immunoprecipitated radiolabelled LipL21 (lane 2). The positions of standard molecular mass markers (in kilodaltons) are indicated on the left.

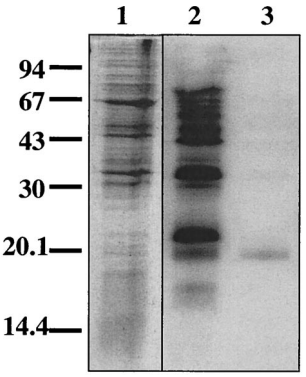

FIG. 5. Blot of biotinylated *L. kirschneri* proteins separated by SDS-PAGE. (Lane 1) A cell lysate of surface biotinylated leptospires stained with Coomassie brilliant blue R-250, (lane 2) a cell lysate from surface biotinylated leptospires probed with horseradish peroxidase-conjugated streptavidin, and (lane 3) LipL21 immunoprecipitated from surface biotinylated leptospires probed with horseradish peroxidase-conjugated streptavidin. The positions of standard molecular mass markers (in kilodaltons) are indicated on the left.

water-soluble reagent Sulfo-NHS-LC-Biotin. After labeling, the residual nonreacting biotinylation reagent was quenched with Tris and removed by several washes before the cells were lysed. When the samples were separated by SDS-PAGE, protein bands of 17, 21, 24, 30, 32, 36, 41, 48, 55, 60, 62, and 70 kDa were selectively biotinylated (Fig. 5). The four most prominently labeled proteins resolved at 21, 24, 32, and 41 kDa; the last two corresponded in mass to the previously characterized OMPs LipL32 and LipL41. When LipL21 was immunoprecipitated with a specific antiserum from a lysate of surface-labeled *L. kirschneri* and separated by SDS-PAGE, a single biotinylated band of 21 kDa was obtained. This band resolved at a mass similar to that of the prominently labeled 21-kDa band from the whole-cell material.

DISCUSSION

In this study, we have characterized LipL21, the second most abundant protein in the *L. interrogans* serovar Lai outer membrane proteome. Previous studies showed no variation in the expression of this protein when leptospires were cultured at 20, 30, or 37°C or at 37°C in the presence of serum or in iron-depleted medium (8). Preliminary data from our laboratories suggested the presence of variations in the relative abundances of leptospiral OMPs between strains (unpublished data). Western blotting with LipL21 polyclonal antisera revealed similar levels of expression of LipL21 among a selection of pathogenic leptospires, with the exception of serovars Bakeri and Fort Bragg, which exhibited slightly lower expression levels. Pathogenic leptospires lose virulence after an undefined number of passages in laboratory culture media and undergo quantitative and qualitative changes in their OMP profiles (22). However, the expression of LipL21 did not appear to vary between the virulent and culture-attenuated strains included in the Western blotting experiments. The uniform expression of LipL21 may indicate that this protein endows the pathogenic members of the genus *Leptospira* with a selective advantage both in the mammalian host and in laboratory cultures.

Southern hybridization and Western blotting experiments did not indicate the presence of the *lipL21* gene or a protein antigenically similar to LipL21 in the nonpathogenic leptospires. An alignment of the LipL21 sequences from six strains (encompassing three species) of pathogenic *Leptospira* revealed 96 to 100% identity, indicating that LipL21 is a new member of the small but growing family of conserved leptospiral OMPs that are unique to the pathogenic strains and that have no significant similarity with proteins of other organisms (including the other pathogenic spirochetes). Other conserved leptospiral OMPs include the porin OmpL1 and the lipoproteins LipL32 and LipL41. Given that these proteins are unique to the pathogenic leptospires, it is likely that they play a specific role in leptospiral pathogenesis. Although it is interesting to speculate about the functions of these proteins, without the technology to generate isogenic mutants of pathogenic *Leptospira* species, definitive studies cannot be performed.

A study investigating the expression of leptospiral antigens during infection identified an antigen that had a molecular mass similar to that of LipL21 and that was designated p22 (4). p22 was observed to react with sera from hamsters challenged with culture-derived leptospires and not with sera from hamsters challenged with host-derived leptospires. In a later study investigating the humoral immune response to leptospirosis, p22 was found to react with pooled human sera from Brazilian leptospirosis patients (15). In our study, sera from hamsters challenged with both host- and culture-derived leptospires were found to react with recombinant LipL21. However, p22 was observed to react only with sera from hamsters challenged with culture-derived leptospires, raising the question of whether p22 and LipL21 are the same. The observation that p22 and LipL21 resolve at approximately the same isoelectric point and molecular mass during two-dimensional electrophoresis suggests that they are the same and that the experimental discordance is due to variations in antibody responses between hamsters. When a random selection of serum samples from MAT-confirmed leptospirosis patients was used in Western blotting experiments, serum samples from two of eight patients recognized recombinant LipL21. The variable patterns of LipL21 recognition observed in humans may reflect differences in the time of collection of sera after the initial infection, the severity of the infection, the genetic heterogeneity of the human host, or even variable masking of LipL21 by other leptospiral surface components. The recognition of recombinant LipL21 by serum samples from leptospirosis patients and hamsters challenged with host-derived organisms suggests that LipL21 is expressed during infections of both hamsters and humans.

In eubacteria, lipoproteins are synthesized with an N-terminal hydrophobic signal peptide that is cleaved from the mature polypeptide by LSP prior to covalent linkage of fatty acids (usually palmitate) (24). The putative LSP recognition site of LipL21 ($I_{-4}F_{-3}A_{-2}A_{-1}C_{+1}$) differs from the spirochetal consensus sequence (16) at positions -4 and -2 and differs from the *E. coli* consensus sequence (41) at position -3. Thus, the LSP recognition site of LipL21 resembles more closely that of *E. coli*. However, when whole cells were grown in the presence of labeled palmitic acid, the 21-kDa band was one of the four most abundant labeled proteins, suggesting that the variation in the LSP recognition site sequence did not affect the effi-

ciency of LipL21 lipidation. The abundant 21-kDa band from the whole-cell material was shown to be LipL21 by immunoprecipitation of a labeled protein with the same molecular mass by LipL21 antiserum.

To examine the cellular location of LipL21 in *Leptospira*, two techniques based on different rationales were used. The first method involves solubilization of the leptospiral outer membrane in situ with TX-114 and the subsequent removal of released periplasmic proteins by phase partitioning. This technique has been used successfully numerous times for a number of spirochetal membrane proteins (9, 22, 40, 43). However, on one occasion the TX-114 method was shown to selectively release a treponemal cytoplasmic membrane lipoprotein (35). Concerns that the TX-114 procedure was releasing cytoplasmic membrane proteins resulted in the development of an alternative procedure for leptospiral membrane fractionation (20). This technique relies on the generation of outer membrane vesicles by alkaline plasmolysis and separation by sucrose density ultracentrifugation. A comparison of the TX-114 detergent fractions and the outer membrane vesicle fractions by two-dimensional electrophoresis revealed similar protein profiles (20), suggesting that both methods result in relatively pure outer membrane fractions. Cellular fractionation with these methods demonstrated the presence of LipL21 in the TX-114 detergent phase and outer membrane vesicle fractions, which were both shown to be free from cytoplasmic membrane markers (Fig. 2). These results unequivocally demonstrate that LipL21 is a component of the leptospiral outer membrane.

Interestingly, during membrane fractionation, virtually no LipL21 could be detected in the cytoplasmic membrane fraction, unlike OMPs LipL36 and LipL41. In the *E. coli* background, it is thought that the amino acid directly after the cysteine of the LSP recognition site sequence determines to which membrane the lipoprotein will localize, presumably by its interaction with the lipoprotein shuttle LolA (37). The amino acids aspartic acid, proline, and tryptophan at this position are thought to cause retention in the cytoplasmic membrane, while phenylalanine, glycine, and tyrosine serve as ambiguous signals, with the resultant lipoprotein being distributed in both the cytoplasmic and the outer membranes. The two amino acids following the cysteine in the LipL21 sequence are both serines, as is also the case in the major outer membrane lipoprotein of *E. coli* (33). The +2 amino acids in the LipL36 and LipL41 sequences are lysine and alanine; by default, these would be trafficked to the outer membrane in an *E. coli* background. However, both the outer membrane and the cytoplasmic membrane fractions contain LipL36 and LipL41, suggesting that lysine and alanine may serve as ambiguous sorting signals in *Leptospira*. The TX-114 method also reveals some LipL41 associated with the protoplasmic cylinder; however, LipL36 appears to be completely absent from this fraction. This anomaly may be due to variability in the solubility of different proteins in TX-114, where proteins that are poorly solubilized in TX-114 are partially extracted and therefore appear in both the cytoplasmic membrane and the outer membrane fractions. The experimental findings of this study suggest that although the TX-114 cellular fractionation technique may provide an accurate representation of the constituents of the outer membrane, this technique does not provide an accurate account of which OMPs may also be represented in the cytoplasmic membrane. When this variability between the two cellular fraction techniques is taken into account, the novel lipoprotein LipL21 appears to be exclusively located in the outer membrane of *Leptospira*.

The identification of proteins located on the surface of the cell is of the utmost importance, as they are likely to be involved in the interaction of leptospires with the external environment, including host cells or extracellular matrix components. In addition, leptospiral surface molecules may serve as targets for immune-mediated clearance. We have shown that LipL21 is exposed on the leptospiral surface by virtue of its susceptibility to the biotinylation of intact cells (Fig. 5). This experimental approach has some clear advantages in determining surface exposure of proteins. Because a water-soluble biotinylation reagent was used, the labeling reaction could be carried out in the absence of organic solvents, such as those which were used in other studies (31) and which may permeabilize the outer membrane. Subsequent immunoprecipitation of the labeled proteins, after quenching of the biotinylation reaction, demonstrates specific labeling of the protein under investigation. Only two other leptospiral proteins have definitively been shown to be exposed on the leptospiral cell surface. The rare outer membrane porin OmpL1 was shown to have surface-exposed epitopes by immunogold labeling of whole cells with specific OmpL1 antiserum (17), while surface immunoprecipitation was used to demonstrate the surface exposure of LipL41 (39). Quizzically, when the surface immunoprecipitation method was applied to LipL36, it appeared not to be surface exposed, although it has been definitively localized to the outer membrane. Experiments visualizing freeze-fractured liposomes have suggested that spirochetal lipoproteins take up a conformation external to the membrane bilayer (27), yet specific proteins seem to have a bias for the inner or outer leaflet of the outer membrane. Nothing is known of the topological mechanisms that orchestrate this process in *Leptospira*; however, a run of six negatively charged residues at the LipL36 N terminus (16) may direct this protein to the inner leaflet of the outer membrane. These residues are notably absent from the surface-exposed proteins LipL21 and LipL41. Future work will investigate whether LipL21 can stimulate protective immunity to leptospirosis.

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