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Molecular Cloning and Sequence Analysis of the Gene Encoding LipL41, a Surface-Exposed Lipoprotein of Pathogenic *Leptospira* Species

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We report the cloning of the gene encoding a surface-exposed leptospiral lipoprotein, designated LipL41. In a previous study, a 41-kDa protein antigen was identified on the surface of *Leptospira kirschneri* (D. A. Haake, E. M. Walker, D. R. Blanco, C. A. Bolin, J. N. Miller, and M. A. Lovett, Infect. Immun. 59:1131–1140, 1991). We obtained the N-terminal amino acid sequence of a staphylococcal V8 proteolytic-digest fragment in order to design an oligonucleotide probe. A Lambda ZAP II library containing *Eco*RI fragments of *L. kirschneri* DNA was screened, and a 2.3-kb DNA fragment which contained the entire structural *lipL41* gene was identified. The deduced amino acid sequence of LipL41 would encode a 355-amino-acid polypeptide with a 19-amino-acid signal peptide, followed by an L-X-Y-C lipoprotein signal peptidase cleavage site. A recombinant His_6-LipL41 fusion protein was expressed in *Escherichia coli* in order to generate specific rabbit antiserum. LipL41 is solubilized by Triton X-114 extraction of *L. kirschneri*; phase separation results in partitioning of LipL41 exclusively into the detergent phase. At least eight proteins, including LipL41 and the other major Triton X-114 detergent phase proteins, are intrinsically labeled during incubation of *L. kirschneri* in media containing [^{3}H] palmitate. Processing of LipL41 is inhibited by globomycin, a selective inhibitor of lipoprotein signal peptidase. Triton X-100 extracts of *L. kirschneri* contain immunoprecipitable OmpL1 (porin), LipL41, and another lipoprotein, LipL36. However, in contrast to LipL36, only LipL41 and OmpL1 were exposed on the surface of intact organisms. Immunoblot analysis of a panel of *Leptospira* species reveals that LipL41 expression is highly conserved among leptospiral pathogens.

Pathogenic spirochetes belonging to the genus *Leptospira* are highly invasive bacteria capable of infecting a broad range of mammalian hosts. Transmission occurs either through direct contact with an infected animal or through indirect contact with soil or water contaminated with urine from a host with chronic renal infection. In humans, leptospirosis occurs sporadically in temperate climates but is a common zoonosis in tropical regions. Leptospirosis is generally a self-limited, febrile illness, but it can progress to hepatic and renal dysfunction and death in 5 to 10% of cases (20). Commercially available vaccines, which consist of heat- or formalin-killed leptospires, produce incomplete, short-term immunity (47). For *Leptospira interrogans* serovar *hardjo*, one of the most prevalent leptospiral serovars in North America, the U.S. Department of Agriculture-licensed vaccine has been shown to be ineffective in the prevention of renal infection in cattle challenged by the conjunctival route (6–8). Thus, there is an important need for development of an improved leptospiral vaccine.

Little is known about surface proteins of pathogenic *Leptospira* species or the pathway(s) by which proteins are exported to the leptospiral surface. Proteins exposed on the cell surface where bacteria interact with the host are of interest as vaccine candidates. We have previously described OmpL1, an outer membrane protein (OMP) that functions as a porin (23, 42). OmpL1 belongs to a class of transmembrane OMPs that have multiple transmembrane segments and surface-exposed loops. A second class of OMPs are the lipoproteins, the polypeptide portions of which are generally hydrophilic and do not contain transmembrane segments (39). Lipoproteins are membrane anchored by fatty-acid residues covalently linked to the N-terminal cysteine (28). The L-X-Y-C lipoprotein signal peptidase cleavage site indicates lipid modification and the capacity for membrane integration, but it is not possible to determine the export pathway or cellular localization of a lipoprotein solely on the basis of sequence data (39). Further information is needed to determine whether a lipoprotein is located exclusively on the subsurface or is exposed on the external leaflet of the outer membrane.

Several surface-exposed lipoproteins have been shown to be protective immunogens in animal models of bacterial diseases (16, 22, 30, 52). Some of the most abundant proteins in spirochetes are lipoproteins. Lipoproteins have been identified to be present in *Treponema pallidum* (12, 13), *Treponema denticola* (35), *Treponema phagedenis* (55), *Serpulina hyodysenteriae* (49), *Borrelia burgdorferi* (9), and the relapsing-fever borreliae (11). To our knowledge, lipoproteins have not been previously described for *Leptospira* species. In this paper, we describe eight lipoproteins that are specifically labeled by incubation of *Leptospira kirschneri* in media containing [^{3}H]palmitate. We also describe the molecular cloning and sequence analysis of one of these lipoproteins, LipL41, which is exposed on the leptospiral surface.

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MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *L. kirschneri* RM52 (48) (formerly *Leptospira alstoni* [23]), and other *Leptospira* species were received from C. A. Bolin (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa) and passaged in Johnson-Harris bovine serum albumin–Tween-80 medium (Bovuminar PLM-5 Microbiological Media; Intergen). *Escherichia coli* DH5α (*supE44* Δ*lacU169* [ϕ80 *lacZ* Δ*M15*] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used as the host strain for transformations of recombinant DNA. *E. coli* PLK-F′ (*recA lac mcrA mcrB hsdR gal supE* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* {Tet^r}]) was used as the host strain for infection with the Lambda ZAP II vector (Stratagene). *E. coli* PLK-F′ and the ExAssist helper phage were used for in vivo excision of the pBluescript phagemid (Stratagene). *E. coli* SOLR (e14$^-$[*mcrA*] Δ[*mcrCB-hsdSMR-mrr*]*171 sbcC recB recJ umuC*::Tn*5*[Kan^r] *uvrC lac gyrA96 relA1 thi-1 endA1* λ^R [F′ *proAB lacI*^{q}*ZΔM15*] Su$^-$ [nonsuppressing]) was used as the host strain for replication of the excised pBluescript phagemid from the Lambda ZAP II vector (Stratagene). *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ[*lac-proAB*] F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]) was used as the host strain for the pRSET expression vector (Invitrogen). The DE3 lysogen of *E. coli* JM109 (Promega) was used as the host strain for pET-15b (Novagen). *E. coli* cells were routinely grown in Luria-Bertani (LB) broth or LB agar, unless otherwise stated (43).

Antisera. Antiserum to *L. kirschneri* RM52 was obtained by immunizing a New Zealand White rabbit with a 1-ml 0.1 M phosphate-buffered saline, pH 7.4 (PBS), suspension of 10^9 washed, sonicated organisms mixed with 1 ml of Freund's complete adjuvant given in equally divided amounts at two intramuscular and two subcutaneous sites. The animal was injected after 4 and 8 weeks with 1-ml PBS suspensions of 1.5×10^9 and 5×10^9 organisms, respectively, mixed with 1 ml of Freund's incomplete adjuvant by using the same combined intramuscular-subcutaneous route. Serum was collected 2 weeks after the final injection. Antisera to OmpL1 and LipL36 were prepared as previously described (23). Briefly, New Zealand White rabbits were immunized with purified His_6 fusion proteins, expressed by *E. coli* JM109 (Invitrogen) transformed with the pRSET plasmid (Invitrogen) containing either the *ompL1* or the *lipL36* gene (23, 24).

Gel electrophoresis and immunoblotting. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in final sample buffer (FSB) composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS. Samples for immunoblots to be probed with OmpL1 antiserum were solubilized in FSB containing 8 M urea. Proteins were separated on a 10% gel with a discontinuous buffer system (31) and stained with Coomassie brilliant blue or were transferred to nitrocellulose (Schleicher and Schuell) for immunoblotting. For antigenic detection on immunoblots, the nitrocellulose was blocked with 5% nonfat dry milk in PBS–0.1% Tween-20 (PBS-T), incubated for 1 h with antiserum diluted 1:5,000 (unless otherwise noted) in PBS-T, and probed with donkey anti-rabbit antiserum conjugated to horseradish peroxidase (Amersham). Immunoblots containing immunoprecipitated antigens were probed with protein A conjugated to horseradish peroxidase (Amersham). Antigen-antibody binding was detected by using the Enhanced Chemiluminescence System (Amersham). Blots were incubated in Enhanced Chemiluminescence System reagents for 1 min and then exposed to XAR-5 film (Kodak).

N-terminal amino acid sequencing of staphylococcal V8 proteolytic fragment. LipL41 was obtained by treatment of *L. kirschneri* with Triton X-114 by a modification of the procedure of Haake et al. (25). *L. kirschneri* cells (5×10^{11}) were washed three times in PBS, resuspended in 2.0 ml of distilled water, and added to 60 ml of 1.0 M NaCl in order to obtain salt-altered cells (2). The bacterial suspension was incubated for 6 h at 25°C, and formation of salt-altered cells was monitored by dark-field microscopy. After centrifugation at 20,000 × *g* for 15 min at 15°C, the pellet was resuspended in 60 ml of 10 mM Tris (pH 8)–1 mM phenylmethylsulfonyl fluoride (PMSF)–1 mM iodoacetamide–10 mM EDTA at 4°C. Triton X-114 (Calbiochem) was added to this suspension to yield a final concentration of 1.0%. After incubation at 4°C for 2 h, removal of the insoluble material and phase separation were performed as described previously (25). The detergent phase proteins were precipitated with acetone and separated by SDS-PAGE. A test strip was stained with Coomassie brilliant blue in order to locate the 41-kDa band, which was cut out of the remainder of the gel and loaded onto a second SDS-PAGE gel in the presence of staphylococcal V8 protease at a concentration of 100 μg ml^{-1} (Sigma). The proteins were allowed to migrate into the stacking gel by electrophoresis, the current was disconnected for 45 min, and then electrophoresis was completed. The polypeptide fragments were transferred to Trans-Blot polyvinylidene difluoride protein-sequencing membrane (Bio-Rad, Richmond, Calif.) and submitted to the UCLA Protein Microsequencing Facility. N-terminal amino acid sequence analysis was performed on a Porton 1090-E gas phase sequenator with on-line detection of PTH amino acids.

Southern blot analysis. *L. kirschneri* genomic DNA was prepared by the method of Yelton and Charon (54). Leptospiral DNA was digested with *Eco*RI and electrophoresed in a 1.0% agarose gel. Following depurination, denaturation, and neutralization, the DNA was transferred to a nylon filter (Zeta-Probe; Bio-Rad) by the method of Southern (41). Filters were cross-linked in a UV Stratalinker (Stratagene) and incubated for 30 min at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were prehybridized for 3 h at 37°C in buffer containing 6× SSC, 1× Denhardt's solution, 0.05% sodium PP_i, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml.

A degenerate oligonucleotide probe, 20 bp in length, was synthesized on the basis of the N-terminal amino acid sequence of the polypeptide fragment. The synthetic oligonucleotide was prepared by using an automated oligonucleotide synthesizer (380B; Applied Biosystems, Inc.). The oligonucleotide probe was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega). Unincorporated label was removed with a NucTrap Probe Purification Column (Stratagene). The filters were then hybridized overnight at 37°C with radiolabeled oligonucleotides at a concentration of 10^6 cpm/ml. Filters were washed at 45°C in 3.0 M tetramethylammonium chloride (Aldrich)–50 mM Tris (pH 8.0)–2.0 mM EDTA–1.0% SDS as previously described (53). Washed filters were exposed to XAR-5 film (Kodak) at −80°C.

Cloning and sequencing of the *lipL41* gene. Standard recombinant-DNA procedures were performed as described elsewhere (41). Restriction endonuclease digests were performed as recommended by the suppliers (New England Biolabs and Promega). The *lipL41* gene was cloned from a Lambda ZAP II library of *L. kirschneri Eco*RI DNA fragments as previously described (23). After restriction mapping, appropriate DNA fragments were subcloned into pBluescript KS and sequenced at the UCLA Core DNA Sequencing Facility by the dideoxy chain termination method with fluorescein-labeled dideoxy nucleotides (Applied Biosystems).

DNA sequence analysis. DNA sequence information was analyzed by the DNA Strider program (33). Homology searches were performed with the BLAST, FASTA, and Profilesearch programs, which are in the University of Wisconsin Genetics Computer Group, Inc., package, version 7.0 (18). Secondary-structure predictions were based upon analysis by using the programs PEPPLOT and PLOTSTRUCTURE, which are also in the Genetics Computer Group package.

Immunization with His_6-LipL41 fusion protein. An 842-bp *Hae*III-*Cla*I fragment of the *lipL41* gene, encoding the amino-terminal 75% of the mature protein, was ligated into pRSETa (Invitrogen) digested with *Pvu*II and *Cla*I. The resulting construct, pRSETa-800HC, was transformed into *E. coli* JM109 (Invitrogen). Expression of the His_6-LipL41 fusion protein was achieved by isopropylthio-β-D-galactoside (IPTG) (Sigma) induction followed by infection with M13/T7 phage containing the T7 polymerase gene driven by the *E. coli lac* promoter. The His_6-LipL41 fusion protein was purified by affinity chromatography by using Ni^{2+}-NTA-Agarose (Qiagen). Antiserum to LipL41 was prepared by immunizing a New Zealand White male rabbit with the purified His_6-LipL41 fusion protein as described previously (23).

Triton X-114 extraction of *L. kirschneri*. *L. kirschneri* cells were extracted with 1% Triton X-114 by a modification of a method described previously (25). In brief, log-phase *L. kirschneri* cells were washed twice in PBS–5 mM $MgCl_2$ and extracted in the presence of 1% protein grade Triton X-114 (Calbiochem)–10 mM Tris (pH 8)–1 mM phenylmethylsulfonyl fluoride (PMSF)–1 mM iodoacetamide–10 mM EDTA at 4°C. The insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. The Triton X-114 concentration of the supernatant was increased to 2%. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 10 min at 2,000 × *g*. The detergent-phase and aqueous-phase proteins were washed and precipitated with acetone.

[^{3}H]palmitate radiolabeling and immunoprecipitation of native LipL41. A 35-ml culture containing 5×10^7 *L. kirschneri* cells per ml in the logarithmic phase of growth was intrinsically labeled by addition of [9,10(*n*)-^{3}H]palmitate (250 μCi; 60 Ci/mmol; Amersham) followed by further incubation in a shaker incubator at 30°C for 48 h until the bacterial concentration reached 1×10^9/ml. Organisms were washed in 5 mM $MgCl_2$ in PBS. A sample for immunoprecipitation containing 8×10^9 *L. kirschneri* cells was resuspended in 1.25 ml of 10 mM Tris HCl (pH 8.0)–10 mM EDTA–1 mM PMSF. To this suspension was added 12.5 μl of 10% protein grade Triton X-100 (Calbiochem), and then the mixture was gently agitated for 30 min at 4°C. The insoluble material was removed by centrifugation at 16,000 × *g* for 10 min. To the supernatant was added 0.2 ml of heat-inactivated (56°C for 30 min) LipL41 rabbit antiserum and 0.25 ml of a slurry of staphylococcal protein A-Sepharose CL-4B (Sepharose-SpA) (Sigma). The suspension was gently agitated for 1 h. The Sepharose-SpA–antibody–antigen complexes were washed twice in 0.01% Triton X-100 in 10 mM Tris HCl, pH 8.0, and resuspended in FSB.

Expression of LipL41 in *E. coli*. A 2.1-kb *Bsp*HI-*Bam*HI DNA fragment encoding the entire LipL41 protein, including its signal peptide, was ligated into pET-15b (Novagen), digested with *Nde*I and *Bam*HI. The resulting construct, pET-15b-L41, was transformed into *E. coli* JM109(DE3) (Promega). Expression of LipL41 was achieved by IPTG (Sigma) induction. After induction, samples were separated by SDS-PAGE and probed with LipL41 antiserum. In experiments involving globomycin inhibition of processing, globomycin (dissolved in ethanol [gift of D. R. Akins]) at a final concentration of 200 μg/ml was added immediately prior to addition of IPTG. The final ethanol concentration was 2%; control experiments without globomycin used ethanol at the same concentration.

Surface immunoprecipitation. Surface immunoprecipitation was performed by a modification of the method of Hansen et al. (27). A 30-ml volume of leptospiral culture containing 6×10^{10} bacteria (>99% actively motile) was

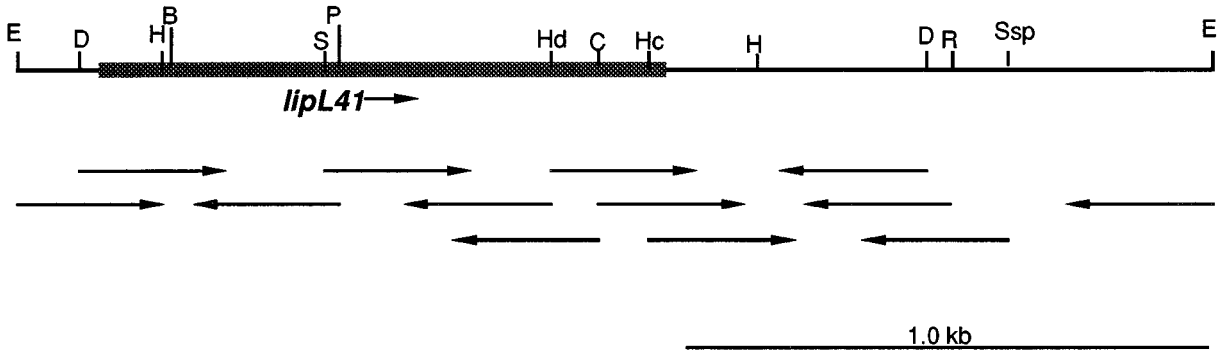

FIG. 1. Partial restriction map of the 2.3-kb *Eco*RI fragment containing the *lipL41* gene and strategy for determining the nucleotide sequence. The *lipL41* gene is 1,065 bp in length; its location is indicated by the shaded region. The arrows below the map indicate the direction and extent of sequence analysis. Single letters above the map indicate the following restriction enzymes: *Eco*RI (E), *Dra*I (D), *Hae*III (H), *Bsi*HKAI (B), *Sca*I (S), *Pvu*II (P), *Hin*dIII (Hd), *Cla*I (C), *Hin*cII (Hc), *Rsa*I (R), and *Ssp*I (Ssp).

mixed with 1.5 ml of heat-inactivated (56°C for 30 min) rabbit antiserum to *L. kirschneri* RM52. The suspension was then gently shaken for 1 h at 30°C. Agglutinated leptospires were pelleted at 2,000 × *g* for 15 min, resuspended in 5 mM $MgCl_2$ in PBS, pelleted again at 2,000 × *g* for 15 min, and then resuspended in 9 ml of 10 mM Tris HCl (pH 8.0)–2 mM EDTA–1 mM PMSF. To this suspension was added 1 ml of 10% protein grade Triton X-100 (Calbiochem), and then the mixture was agitated for 30 min at 4°C. The insoluble material was removed by centrifugation at 16,000 × *g* for 20 min. To the supernatant were added 1 ml of 2% deoxycholate, 50 μl of 20% SDS, and 600 μl of a slurry of Sepharose-SpA (Sigma). This mixture was gently agitated for 30 min at 4°C. The Sepharose-SpA–antibody–antigen complexes were washed twice in 0.01% Triton X-100 in 10 mM Tris HCl (pH 8.0), and resuspended in FSB. Samples were then processed by SDS-PAGE and immunoblotting with antisera specific for OmpL1, LipL36, and LipL41. An immunoprecipitation control experiment was performed in parallel under the same conditions as those described above except that immunoprecipitation was performed after solubilization in Triton X-100. Immunoprecipitation of Triton X-100-solubilized antigens was performed as described above for radiolabeled LipL41 with the following exceptions: (i) immunoprecipitation involved the use of rabbit antiserum raised against *L. kirschneri* RM52, and (ii) Sepharose-SpA (Sigma) was added in the presence of 0.2% deoxycholate and 0.1% SDS.

Nucleotide sequence accession number. The nucleotide sequence of the *lipL41* gene from *L. kirschneri* RM52 has been deposited in the GenBank database under the accession number L46794.

RESULTS

Design of oligonucleotide probes and cloning of the *lipL41* gene. Staphylococcal V8 protease digestion of LipL41 resulted in fragments with molecular masses of 21 and 17 kDa. N-terminal amino acid sequence analysis of the 17-kDa fragment revealed the sequence ASLSLTGITKNRAKIGNL. A 20-bp oligonucleotide probe with 864-fold degeneracy, AC(TAG)G G(TAG)AT(CAT)AC(TCAG)AA(AG)AA(TC)(AC)G, was designed on the basis of the TGITKNR portion of the sequence. On the basis of the low GC content of *Leptospira* spp. (29) codon bias was used for the first threonine residue and the glycine residue. The oligonucleotide probe identified a 2.3-kb *Eco*RI fragment by Southern hybridization of the *L. kirschneri* genome (data not shown). The 2.3-kb *Eco*RI fragment was cloned from a partial Lambda ZAP II (Stratagene) library of *L. kirschneri* genomic DNA that was described previously (23).

Sequence analysis of the *lipL41* gene. Restriction mapping, Southern blot analysis, and DNA sequencing revealed that the entire *lipL41* gene is encoded by the 2.3-kb *Eco*RI fragment (Fig. 1). An intact open reading frame was identified 170 bp downstream from the *Eco*RI site. The *lipL41* structural gene consists of 1,065 bases encoding a protein of 355 amino acids (Fig. 2). Sigma 70-like −35 (TTGACA) and −10 (TTAAAT) promoter regions are present upstream from the initiation codon and are spaced 17 bp apart, which is consistent with their interaction with RNA polymerase on the same side of the double helix (43). A potential ribosome-binding site (AGGA) is also present upstream from the initiation codon.

As expected for a lipoprotein, the deduced amino acid sequence begins with a 19-residue signal peptide (Fig. 2). The sequence of the LipL41 signal peptide conforms to the rules established for procaryotic lipoprotein signal peptides (28, 39). The LipL41 signal peptide has a basic amino-terminal region (including an arginine at position 2 and a lysine at position 3), a hydrophobic core (amino acids 4 through 17), and a carboxy-terminal Leu-X-Y-Cys lipoprotein signal peptidase cleavage site. The hydrophobicity of the signal peptide core region is reflected by the N-terminal peak of 3.5 on the Kyte-Doolittle hydrophobicity plot (Fig. 3). Following the signal peptide, there are no long alpha-helical hydrophobic membrane-spanning domains as found in transmembrane cytoplasmic membrane proteins.

After cleavage of the 19-amino-acid signal peptide by leptospiral lipoprotein signal peptidase, the mature polypeptide would have a predicted molecular mass of 36.8 kDa. Immediately following the glutamic acid residue 104 is a sequence of 18 amino acids that is identical to the sequence obtained by N-terminal amino acid sequence analysis of the 17-kDa staphylococcal V8 proteolytic digest fragment of the native protein (Fig. 2). Staphylococcal V8 protease is known to cleave peptides following acidic amino acids (19). At a location 27 bp downstream from the termination codon is an inverted repeat followed by five T residues, a configuration observed for rho-independent transcription terminators (Fig. 2).

Secondary-structure predictions according to both Chou-Fasman and Garnier-Osguthorpe-Robson rules predict that 60% of the mature LipL41 protein is alpha-helical. The alpha-helical conformation could be stabilized by the presence of 22 potential salt bridges conforming to the N+4 rule (34). Database searching by using the FASTA, BLAST, and ProfileSearch programs failed to reveal significant amino acid homologies. However, comparison of the LipL41 amino acid sequence with those of 20 other spirochetal lipoprotein sequences revealed carboxy-terminal homology with 5 that are either surface exposed or outer membrane associated (Fig. 4) (1, 3, 26, 32). Alignment of the LipL41 sequence with the OspA sequence of *B. burgdorferi* by using the GAP program revealed 53% identity in the carboxy-terminal 15 residues of the alignment. Of the eight *T. pallidum* lipoproteins for which the complete sequences have been reported, the carboxy terminus of TmpA was most homologous with LipL41 (36). In-

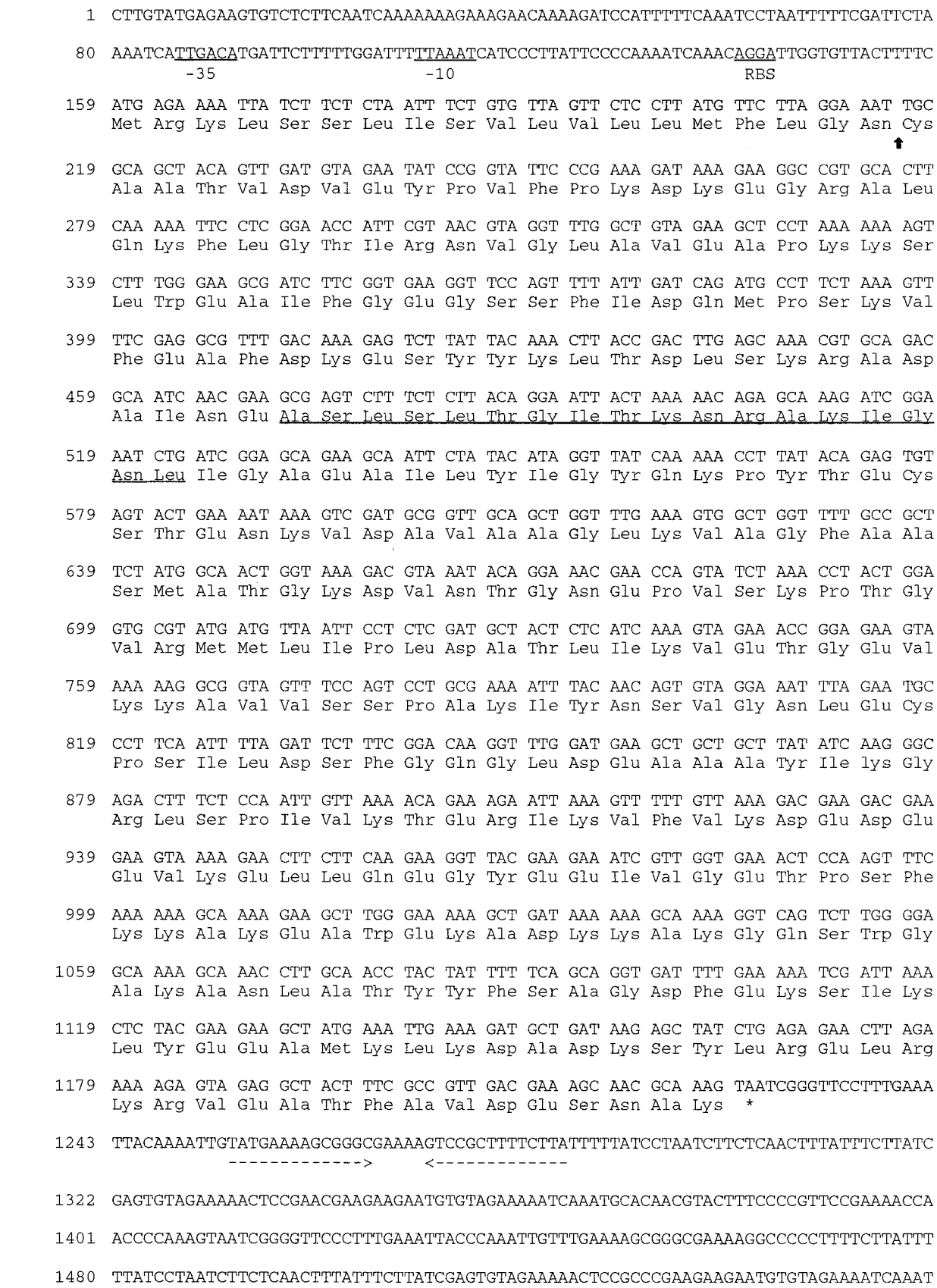

FIG. 2. Nucleotide sequence and deduced amino acid sequence of *lipL41*. Putative sigma 70 −35 and −10 promoter regions and a ribosome-binding site (RBS) are shown. The putative lipoprotein signal peptidase cleavage site is indicated by a vertical arrow. The amino acid sequence obtained from the staphylococcal V8 protease digestion of the native protein is underlined. The location of the TAA stop codon is indicated by an asterisk. An inverted repeat, which may function as a rho-independent transcription terminator, is indicated by the horizontal broken arrows.

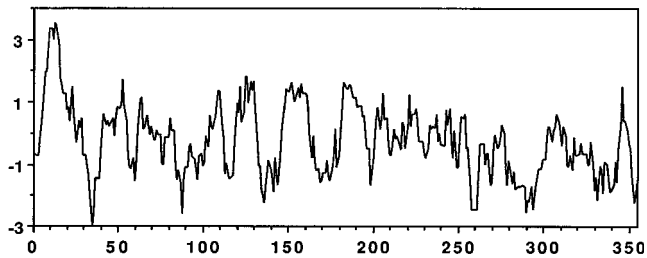

FIG. 3. Kyte-Doolittle hydrophobicity plot of the derived amino acid sequence of LipL41. The N-terminal peak corresponds to the signal peptide. No other significant hydrophobic peaks are found in the hydrophobicity plot, indicating that LipL41 is not a transmembrane cytoplasmic membrane protein.

terestingly, it has recently been reported that TmpA is a component of the *T. pallidum* outer membrane (4). The carboxy terminus of a *Campylobacter jejuni* lipoprotein was also homologous to LipL41 (38).

Behavior of LipL41 during Triton X-114 extraction and phase partitioning. We analyzed the behavior of LipL41 in the nonionic detergent Triton X-114 and tested the specificity of the LipL41 antiserum. Triton X-114 extraction of *L. kirschneri* solubilizes the leptospiral outer membrane, including the porin, OmpL1 (23, 25). Immunoblots with LipL41 antiserum of *L. kirschneri* fractionated with Triton X-114 revealed reactivity in the whole organism and the detergent phase fraction, but not the aqueous-phase fraction (Fig. 5). Selective partitioning into the Triton X-114 detergent phase is a known characteristic of membrane lipoproteins. Solubilization of LipL41 by Triton X-114 was incomplete, a pattern that was also observed for the outer membrane porin OmpL1 (23). The amphiphilic behavior of LipL41 is consistent with modification by fatty acids. This is confirmed by the finding that the His_6-LipL41 fusion protein was found to be highly soluble in aqueous buffers lacking detergent (data not shown).

***L. kirschneri* acylates LipL41.** Intrinsic labeling of *L. kirschneri* with [^{3}H]palmitate resulted in acylation of at least eight proteins (Fig. 6). These proteins have been designated LipL32, LipL36, LipL41, LipL46, LipL49, LipL53, LipL59, and LipL71. Comparison with a Coomassie blue-stained gel run under the same conditions showed that protein labeling was selective and included five prominent Triton X-114 detergent phase proteins, LipL36, LipL41, LipL46, LipL49, and LipL59. Immunoprecipitation of a Triton X-100 extract of [^{3}H]palmitate-labeled *L. kirschneri* by using LipL41 antiserum confirmed that LipL41 is one of the proteins acylated by *L. kirschneri*. The gene encoding LipL36 has been cloned, and its deduced amino acid sequence also contains an N-terminal signal peptide with an L-X-Y-C lipoprotein signal peptidase cleavage site (24). LipL32 may be the 31-kDa major membrane protein of *L. interrogans* serovar pomona, which was found to be heat labile through the activity of an endogenous cysteine protease (56). Both the 31-kDa major membrane protein and LipL32 are lost during phase partitioning of the Triton X-114 extract, which involves warming the extract to 37°C (Fig. 6). Intrinsic labeling of *L. kirschneri* also resulted in incorporation of label into leptospiral lipopolysaccharide, which appears diffusely at the bottom of the lane (25).

Globomycin inhibits processing of LipL41. In order to demonstrate that LipL41 is processed by lipoprotein signal peptidase, *E. coli* JM109(DE3) cells containing pET-15b-L41 were treated with various concentrations of IPTG with or without globomycin, a selective inhibitor of lipoprotein signal peptidase. LipL41 was not expressed in the absence of IPTG. The majority of LipL41 expressed at IPTG concentrations at or above 0.04 mM was unprocessed, with an apparent molecular mass about 2 kDa greater than that of the processed form (Fig. 7, lane 2). The apparent molecular mass of the processed form of LipL41 was identical to that of the native protein (data not shown). When the concentration of IPTG was lowered to 0.01 mM, most of the LipL41 observed was processed, primarily because there was less of the unprocessed form (Fig. 7, lane 1). Induction in the presence of globomycin inhibited processing of LipL41, resulting in an increase in the unprocessed form and a decrease in the processed form (Fig. 7, lane 3). These data indicate that the LipL41 signal peptide is processed by *E. coli* lipoprotein signal peptidase. The observation that processing was incomplete at higher IPTG concentrations suggests that the LipL41 signal peptide is processed more efficiently in *L. kirschneri* than in *E. coli.*

LipL41 is exposed on the surface of *L. kirschneri*. Previous surface immunoprecipitation studies identified the rare outer membrane porin OmpL1 and two other protein antigens with molecular masses of 41 and 45 kDa (25). Specific immunological reagents were not available when the prior study was performed (25). In order to confirm that LipL41 is exposed on the surface of *L. kirschneri*, we reexamined the surface immunoprecipitation technique using antisera specific for OmpL1, LipL36, and LipL41. In this procedure, antiserum raised against whole *L. kirschneri* is allowed to bind to motile, intact organisms. Unbound antibody is removed prior to solubilization and immunoprecipitation. The prior study also lacked an immunoprecipitable subsurface antigen as a negative control for the integrity of the outer membrane. In order to evaluate the specificity of the surface immunoprecipitation technique for surface antigens, the present study evaluated immunoprecipitation both before and after Triton X-100 extraction. The surface and Triton-soluble immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with antisera specific for OmpL1, LipL36, and LipL41. As shown in Fig. 8, OmpL1, LipL41, and large amounts of LipL36 were immunoprecipi-

```
                                          *  * | * |* | *      | *  **|  || |
Leptospira kirschneri  LipL41     MRKLSSLISVLVLLMFLGNCAAT...VEATFAVDE.SNA.K
Borrelia burgdorferi   OspA          MKKYLLGIGLILALIACKQN...VEIT.KLDEIKNALK
Borrelia burgdorferi   OspB           MRLLIGFALALALIGCAQK...SEIK.NLSELKNALK
Borrelia burgdorferi   OspE       MNKKNKNFIVYAVFILIGACKIH...AEYAISLEELKKNLK
Treponema pallidum     TmpA    MNAHTLVYSGVALACAAMLGSCASG...SPKSSMNEE..GASR
Treponema phagedenis   TmpA    MKLKSLVFSLSALFLVLGFTGCKSK...IESTEEPIE..GGVQ
Campylobacter jejuni   Hemolysin MKKSLVFAFFAFFLSLILTACNSN...ESLKTMILEIKNAVK
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FIG. 4. Amino acid similarities between LipL41 and other bacterial lipoproteins. The lipoprotein signal peptidase cleavage site (underlined) and the carboxy terminus of LipL41 are homologous to a number of other lipoproteins, most of which have been shown to be either surface exposed or outer membrane associated. The amino- and carboxy-terminal sequences of each lipoprotein are shown. Dots indicate locations of sequence gaps introduced in order to optimize the alignment with LipL41. Locations are indicated where the LipL41 residues are identical (|) or functionally similar (*) to at least half of those in the other six lipoproteins, according to the mutation matrix of Dayhoff et al. (17).

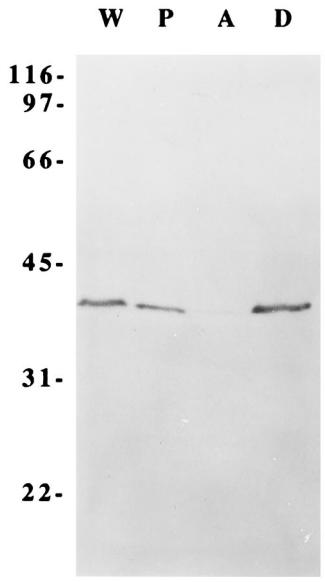

FIG. 5. Behavior of LipL41 in Triton X-114 and specificity of LipL41 antiserum. Triton X-114 fractions of *L. kirschneri* organisms were separated by SDS-PAGE and probed with LipL41 antiserum. Fractions analyzed were the whole organism (W) and Triton X-114-insoluble pellet (P), aqueous-phase (A), and detergent phase (D) material. The numbers of cell equivalents were the same in all four lanes. The locations of the molecular size standards are shown (in kilodaltons) on the left. Solubilized LipL41 partitions selectively into the Triton X-114 detergent phase.

tated from Triton X-100-solubilized extracts. Only a trace of LipL36 was found in the surface-immunoprecipitated material, most likely reflecting a small percentage of disrupted organisms. In contrast, OmpL1 and LipL41 were accessible to antibody on the surface of intact *L. kirschneri*. These results indicate that OmpL1 and LipL41 are surface exposed while LipL36 is not surface exposed.

Expression of LipL41 in *Leptospira* species. To determine the level and distribution of LipL41 expression, immunoblot analysis was performed with a panel of *Leptospira* species, with antiserum from a rabbit immunized with purified His_6-LipL41 (Fig. 9). The LipL41 antiserum is reactive with a single band with a molecular mass of 41 kDa. The molecular weight and amount of LipL41 expressed among pathogenic *Leptospira* species are highly conserved. LipL41 is expressed in relatively the same amounts by all leptospiral pathogens tested. There was a positive correlation between leptospiral pathogenicity and reactivity with antiserum to LipL41. Although there was weak reactivity in *Leptospira inadai*, no 41-kDa antigens were detected in two other nonpathogenic *Leptospira* species, *Leptospira biflexa* and *Leptospira wolbachii*, or in the related nonpathogen *Leptonema illini*. The significance of the lower-molecular-weight antigen in *L. wolbachii* remains to be determined.

DISCUSSION

LipL41 was identified during a prior study, the objective of which was to define the surface antigens of virulent and culture-attenuated *L. kirschneri* RM52 (25). In those surface immunoprecipitation experiments, three proteins with apparent molecular masses of 31, 41, and 45 kDa were identified. The gene encoding the 31-kDa protein was subsequently cloned and sequenced, allowing characterization of its gene product, OmpL1, as a rare leptospiral outer membrane porin (23, 42). Like other porin proteins, OmpL1 is assumed to have a number of surface-exposed regions and OmpL1 surface epitopes have been demonstrated by immunoelectron microscopy (23). Although OmpL1 is a minor protein in comparison with the total *L. kirschneri* protein profile, the surface immunoprecipitation technique correctly identified it as an important surface component. This validation of the sensitivity of the surface immunoprecipitation technique for *Leptospira* species increased our interest in the remaining two protein antigens identified by this technique, with apparent molecular masses of 41 and 45 kDa. Surface immunoprecipitation studies by other investigators had also identified a 41-kDa antigen without characterizing it further (37, 56).

Several lines of evidence support the conclusion that LipL41 is modified by lipid at its N-terminal cysteine residue. LipL41 was found to be blocked to N-terminal amino acid sequencing until subjected to staphylococcal V8 protease digestion. Analysis of the deduced amino acid sequence of LipL41 revealed a signal peptide followed by a typical L-X-Y-C lipoprotein signal peptidase cleavage site, homologous to that of known spirochetal lipoproteins (Fig. 4). Sequence analysis is sufficient for discrimination between the signal peptides of lipoproteins and nonlipoproteins (51). Further evidence was obtained from analysis of the His_6-LipL41 fusion protein, which lacks the signal peptide and N-terminal cysteine and is highly soluble in aqueous buffers lacking detergent. In contrast, native LipL41 is amphiphilic, partitioning into the Triton X-114 detergent phase (Fig. 5), consistent with modification by fatty acids.

Intrinsic labeling of *L. kirschneri* with [9,10(*n*)-^{3}H]palmitate

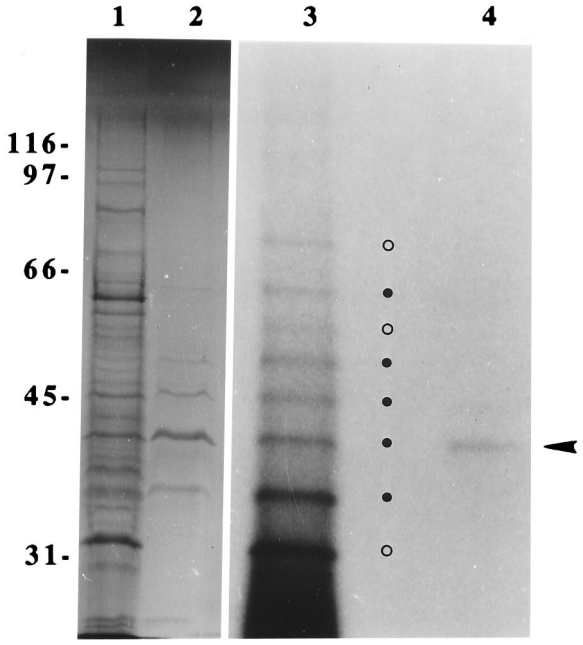

FIG. 6. LipL41 is selectively acylated by *L. kirschneri*. Lanes 1 and 2 show a Coomassie brilliant blue-stained SDS-PAGE gel of *L. kirschneri* total and Triton X-114 detergent phase proteins, respectively. Lanes 3 and 4 show an autoradiogram of *L. kirschneri* proteins intrinsically labeled by [^{3}H]palmitate and separated by SDS-PAGE. Lane 3 contains total *L. kirschneri* proteins. Lane 4 contains material immunoprecipitated by addition of LipL41 antiserum to a Triton X-100 extract of *L. kirschneri*. The locations of the molecular size standards are shown (in kilodaltons) on the left. Intrinsic labeling by incubation of *L. kirschneri* in media containing [^{3}H]palmitate resulted in selective acylation of eight proteins. The solid circles between lanes 3 and 4 indicate the five acylated proteins that are also found in the Triton X-114 detergent phase. The open circles indicate acylated proteins not found in the Triton X-114 detergent phase.

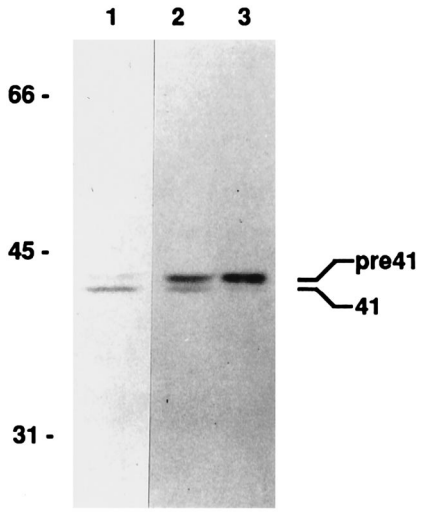

FIG. 7. Globomycin inhibition of LipL41 processing in *E. coli*. *E. coli* JM109(DE3) cells containing pET-15b-L41 were treated with 0.01 mM IPTG without globomycin (lane 1), 0.04 mM IPTG without globomycin (lane 2), or 0.04 mM IPTG with globomycin (lane 3); separated by SDS-PAGE; and probed with LipL41 antiserum. The locations of processed (41) and unprocessed (pre41) LipL41 are shown on the right. The locations of the molecular size standards are shown (in kilodaltons) on the left.

resulted in selective labeling of eight proteins, including LipL41 (Fig. 6). Although *Leptospira* species metabolize fatty acids by beta-oxidation to acetyl coenzyme A (acetyl-CoA), which is a carbon source in the biosynthesis of amino acids, it should be noted that 75% of the tritium label in [9,10(n)-^{3}H]palmitate is transferred to water during the process of beta-oxidation. Tritium label that is metabolized to acetyl-CoA will be diluted in the acetyl-CoA pool prior to incorporation into amino acids. For these reasons, labeling of proteins by incorporation of tritiated amino acids is likely to be relatively inefficient in comparison with modification of lipoproteins by one or more molecules of [^{3}H]palmitate. This point is borne out by the finding that there was selective labeling of leptospiral proteins by [^{3}H]palmitate (Fig. 6). Most of the proteins labeled by [^{3}H]palmitate are also Triton X-114 detergent phase proteins. Correspondence between spirochetal lipoproteins and Triton X-114 detergent phase proteins has also been observed with *T. pallidum* and *B. burgdorferi* (9, 12).

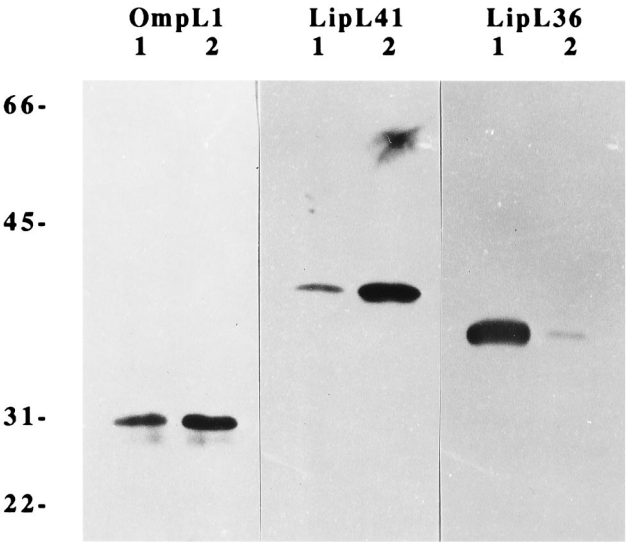

FIG. 8. Immunoprecipitation of OmpL1 and LipL41 on the surface of *L. kirschneri*. Identical samples of material immunoprecipitated with antiserum to *L. kirschneri* were separated by SDS-PAGE for each of the three panels shown and probed with antisera specific for OmpL1, LipL41, and LipL36. Proteins were immunoprecipitated after (lanes 1) and before (lanes 2) solubilization with Triton X-100, in order to identify surface-exposed proteins among those that are detergent soluble. Both LipL41 and the surface-exposed porin OmpL1 were found to be present in the surface-immunoprecipitated material. Although LipL36 was immunoprecipitated by the rabbit antiserum after solubilization by Triton X-100, it was not accessible to antibody on the surface of *L. kirschneri*. The locations of the molecular size standards are shown (in kilodaltons) on the left.

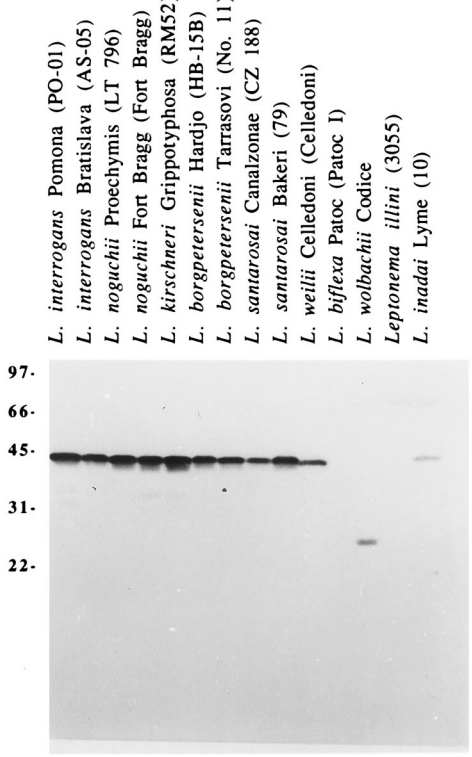

FIG. 9. An immunoblot of a panel of *Leptospira* species obtained by using LipL41 antiserum. LipL41 expression is highly conserved among the pathogenic *Leptospira* species, *L. interrogans*, *L. noguchii*, *L. kirschneri*, *L. borgpetersenii*, *L. santarosai*, and *L. weilii*. *L. biflexa*, *L. wobachii*, and *L. inadai* are nonpathogenic species, as is the related organism *Leptonema illini*. The locations of the molecular size standards are shown (in kilodaltons) on the left.

Further evidence that LipL41 is a lipoprotein was obtained by studying the expression of LipL41 in *E. coli* JM109(DE3) by using the pET-15b-L41 expression vector (Fig. 7). We found that LipL41 signal peptide processing was inhibited by globomycin, a selective inhibitor of lipoprotein signal peptidase. On the basis of these data, LipL41 satisfies three of Wu's criteria for definition of a lipoprotein: (i) it has an L-X-Y-C lipoprotein signal peptidase cleavage site, (ii) there is inhibition of processing by globomycin, and (iii) the protein is labeled by tritiated palmitate (28). Processing of the LipL41 signal peptide appeared to be less efficient in *E. coli* than in *L. kirschneri*. This may be due to the fact that the LipL41 signal peptide cleavage site differs from the LA(G or A)$\downarrow$C consensus sequence (the arrow indicates the cleavage site) in that there is an asparagine at the -1 position, instead of glycine or alanine (51). The only other lipoprotein signal peptide cleavage site

with an asparagine at the −1 position that we are aware of is the *B. burgdorferi* protein S1 (21), suggesting the cleavage site specificities of spirochete lipoprotein signal peptidases differ from that of *E. coli* lipoprotein signal peptidase.

Our identification of the N-terminal cysteine residue of the mature LipL41 molecule is supported by the preceding L-X-Y-C lipoprotein signal peptidase cleavage site and the hydrophobic signal peptide. In addition, *E. coli*-like −35 and −10 sigma 70 promoter regions are located upstream of the putative start codon. Although there is a discrepancy of approximately 4 kDa between the observed mobility of LipL41 and its calculated molecular mass of 36.8 kDa, we believe that we have also correctly identified the TAA stop codon because of the inverted repeat located 27 bp downstream. Internal deletions during the cloning process are unlikely because PCRs using primers specific for the beginning and end of the *lipL41* gene amplify DNA fragments of the same size with either genomic or plasmid DNA as the template (data not shown). Covalent modification of the N-terminal cysteine by lipid would not account for a discrepancy of 4 kDa. The slower-than-predicted electrophoretic mobility of LipL41 may be due to the unusually large number of acidic residues (51 of 336 = 15%) in the mature protein, which could reduce SDS binding. A similar argument has been put forward to explain the discrepancy between observed mobility and calculated molecular mass for the lipoprotein TpN34 of *T. pallidum* (46).

The molecular cloning of the gene encoding LipL41 made it possible to generate antiserum specific for this molecule. Using this immunological reagent, we were able to reexamine the surface immunoprecipitation technique in order to confirm that LipL41 is the same as the 41-kDa surface antigen identified in previous studies. Because of the fragility of the spirochetal outer membrane, it was desirable to control for the specificity of the surface immunoprecipitation technique by using an immunoprecipitable, Triton X-100-soluble, subsurface antigen. Monoclonal antibody probes are available for detection of leptospiral endoflagella (50); however, this structure is not effectively solubilized by Triton X-100 (data not shown). A second lipoprotein, LipL36, a major Triton X-100-soluble leptospiral protein, proved to be an ideal negative control (24). We used LipL36 antiserum to demonstrate that immunoprecipitation of LipL36 requires Triton X-100 solubilization of the outer membrane prior to addition of *L. kirschneri* antiserum (Fig. 8). These results suggest that LipL36 is a subsurface structure. An alternative explanation is that the LipL36 is surface exposed but the epitope(s) recognized by *L. kirschneri* antiserum is not available until denaturation of LipL36 by Triton X-100. We believe the latter explanation is unlikely because the immunoprecipitation antiserum was generated by immunization with native *L. kirschneri* proteins and because enhancement of immunoprecipitation was not observed for either OmpL1 or LipL41.

The strategy of comparing surface-exposed and detergent-soluble antigens makes it possible to address the distribution of antigens between surface and subsurface compartments. This approach has also been used by other investigators to distinguish leptospiral surface proteins from subsurface proteins (37). Nunes-Edwards et al. (37) found that leptospiral surface proteins were a minority of the total population of leptospiral proteins that could be immunoprecipitated after detergent solubilization. Analogous approaches to exposing subsurface antigens by Triton X-100 treatment have been utilized in order to demonstrate that endoflagella and other major antigens of *T. pallidum* are largely subsurface structures (14, 15). Thin-section immunoelectron microscopy and purification of the spirochetal outer membrane are other approaches which have been applied to assessing the distribution of the outer surface proteins A and B in *B. burgdorferi* (5, 10, 40, 44).

Surface immunoprecipitability suggests that some fraction of the LipL41 population is anchored to the external leaflet of the leptospiral outer membrane. Preliminary outer-membrane isolation studies indicate that LipL41 is a leptospiral OMP (data not shown). Additional experiments involving immunoelectron microscopy will be necessary to determine the cellular distribution of LipL41. The homology of the carboxy terminus of LipL41 to those of other surface-exposed and outer-membrane-associated spirochetal lipoproteins raises the intriguing possibility that this region of the molecule is involved in outer-membrane targeting. Studies involving site-directed mutagenesis have shown that the carboxy terminus of nonlipidated transmembrane OMPs is important either in targeting to or insertion into the outer membrane (45). Although the function of LipL41 is not understood, its surface exposure and conservation of expression among pathogenic *Leptospira* species indicate that this molecule merits further study as a potential protective immunogen.

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