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Characterization of Leptospiral Outer Membrane Lipoprotein LipL36: Downregulation Associated with Late-Log-Phase Growth and Mammalian Infection

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We report the cloning of the gene encoding a 36-kDa leptospiral outer membrane lipoprotein, designated LipL36. 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 *Leptospira kirschneri* DNA was screened, and a 2.3-kb DNA fragment which contained the entire structural *lipL36* gene was identified. Several lines of evidence indicate that LipL36 is lipid modified in a manner similar to that of LipL41, a leptospiral outer membrane lipoprotein we described in a previous study (E. S. Shang, T. A. Summers, and D. A. Haake, Infect. Immun. 64:2322–2330, 1996). The deduced amino acid sequence of LipL36 would constitute a 364-amino-acid polypeptide with a 20-amino-acid signal peptide, followed by an L-X-Y-C lipoprotein signal peptidase cleavage site. LipL36 is solubilized by Triton X-114 extraction of *L. kirschneri*; phase separation results in partitioning of LipL36 exclusively into the hydrophobic, detergent phase. LipL36 is intrinsically labeled during incubation of *L. kirschneri* in media containing [^{3}H]palmitate. Processing of LipL36 is inhibited by globomycin, a selective inhibitor of lipoprotein signal peptidase. After processing, LipL36 is exported to the outer membrane along with LipL41 and lipopolysaccharide. Unlike LipL41, there appears to be differential expression of LipL36. In early-log-phase cultures, LipL36 is one of the most abundant *L. kirschneri* proteins. However, LipL36 levels drop considerably beginning in mid-log phase. LipL36 expression in vivo was evaluated by examining the humoral immune response to leptospiral antigens in the hamster model of leptospirosis. Hamsters surviving challenge with culture-adapted virulent *L. kirschneri* generate a strong antibody response to LipL36. In contrast, sera from hamsters surviving challenge with host-adapted *L. kirschneri* do not recognize LipL36. These findings suggest that LipL36 expression is downregulated during mammalian infection, providing a marker for studying the mechanisms by which pathogenic *Leptospira* species adapt to the host environment.

Leptospirosis is an important global human and veterinary health problem caused by spirochetes belonging to the genus *Leptospira*. Human leptospirosis is a potentially fatal disease which appears to be emerging in both developed and underdeveloped regions of the world (11, 42). In domestic animals, leptospirosis is an important cause of abortion, stillbirth, infertility, decreased milk production, and death (41). Leptospires are ubiquitous in nature, reflecting their ability to adapt to both the ambient environment and the renal tubules of chronically infected reservoir hosts. Cattle and feral rodents are the most important reservoir hosts, although pathogenic *Leptospira* species have been isolated from essentially every known mammalian species. Leptospirosis control efforts have also been hampered by the fact that commercially available veterinary vaccines, which consist of inactivated whole-cell bacterins, depend largely on serovar-specific leptospiral lipopolysaccharide (LPS) carbohydrate antigens for their efficacy. This approach has been demonstrated to be ineffective in the prevention of disease in cattle (6–8), and its efficacy in other animals has serious limitations (41). For these reasons, there is an urgent need for development of alternative vaccine strategies relying on an improved understanding of leptospiral outer membrane proteins (OMPs).

The focus of our research has been to identify and characterize OMPs which are relevant in the pathogenesis of leptospirosis. For this reason, we have been interested in studying how levels of OMP expression change when cultivated, virulent leptospires are introduced into a mammalian host. The pathogenic *Leptospira* species *L. interrogans* and *L. kirschneri* and other invasive spirochetes express uniquely low levels of transmembrane OMPs (14, 26, 30, 32, 44, 45). Downregulation of OMP expression may be an important mechanism by which spirochetes evade the host immune response (4, 14, 20, 29, 32, 44, 45). Consistent with this hypothesis, there is a correlation between decreased levels of transmembrane OMPs and pathogenicity in both *L. kirschneri* and *Borrelia burgdorferi* (14, 31). In the case of *L. kirschneri*, this observation was a key to the identification of the rare OMP OmpL1, a surface-exposed leptospiral porin (13, 37). A number of proteins have been shown to be subject to differential expression during the life cycle of *B. burgdorferi*. Expression of the outer surface protein OspA

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and the outer membrane-associated lipoprotein lp6.6 is downregulated when *B. burgdorferi* in the tick midgut infects the mammalian host (3, 22). Expression of other *B. burgdorferi* proteins, including EppA, OspC, OspE, OspF, and pG, is upregulated during mammalian infection (10, 36, 40, 46).

Studies designed to identify OMPs have suggested that some of the most abundant leptospiral proteins are associated with the outer membrane (9, 14, 27, 51). For example, the most prominent protein in the leptospiral total-membrane profile is a 31-kDa protein which is solubilized by extraction of the outer membrane with Triton X-100 (51). These results contrast markedly with the low outer membrane particle density observed by freeze-fracture electron microscopy (14). An explanation for the apparent contradiction between the ultrastructural data and the OMP isolation studies was provided by our subsequent finding that, as in other spirochetes, many of the most abundant leptospiral proteins appear to be lipoproteins which are membrane anchored, not by transmembrane domains, but by fatty acids modifying their amino-terminal cysteine (38). Incubation of *L. kirschneri* in media containing tritiated palmitate resulted in intrinsic labeling of the 41-kDa protein designated LipL41 and the other major hydrophobic, detergent-extractable membrane proteins. Molecular cloning and sequencing of the gene encoding LipL41 revealed a Leu-X-Y-Cys consensus lipoprotein signal peptidase cleavage site. Furthermore, processing of LipL41 was found to be inhibitable by globomycin, a selective inhibitor of lipoprotein signal peptidase. Consistent with fatty acid modification, native LipL41 partitions exclusively into the Triton X-114 hydrophobic, detergent phase (38).

In this report we describe the gene encoding a second leptospiral lipoprotein, LipL36, which differs from LipL41 in its pattern of protein localization and expression. A previous report presented evidence indicating that LipL41 is exported to the outer leaflet of the outer membrane, while LipL36 is restricted to the periplasmic leaflet of the outer membrane (38). In the present study, we evaluated the outer/cytoplasmic (inner) membrane distribution of LipL36 and LipL41 by comparing rates of Triton X-100 solubilization. We also compared in vivo expression of LipL36 and LipL41, providing evidence for differential OMP expression in the leptospiral life cycle.

(Portions of this work were presented at the 95th and 96th General Meetings of the American Society for Microbiology, in Washington, D.C., 21 to 25 May 1995, and in New Orleans, La., 19 to 23 May 1996, respectively.)

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Virulent and culture-attenuated *L. kirschneri* RM52 organisms (1, 14) were passaged in Johnson-Harris bovine serum albumin Tween 80 medium (Bovuminar PLM-5 Microbiological Media; Intergen) (19). *E. coli* DH5α (*supE44* Δ*lacU169* [ϕ80 *lacZ* ΔM15] *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} *lacZ*ΔM15]) 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 on LB agar, unless otherwise mentioned (35).

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. Proteins were separated on a 12% gel with a discontinuous buffer system (21) and stained with Coomassie brilliant blue, or they were transferred to nitrocellulose filters (Schleicher and Schuell) for immunoblotting. For antigenic detection on immunoblots, the nitrocellulose filter was blocked with 5% nonfat dry milk in PBS (0.1 M phosphate-buffered saline, pH 7.4)–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). Antigen-antibody binding was detected with the enhanced chemiluminescence system (ECL; Amersham). Blots were incubated in ECL reagents for 1 min and then exposed to XAR-5 film (Kodak). Densitometry of immunoblots was performed with an AMBIS imager and QuantProbe software (Scanalytics, Inc., Billerica, Mass.).

Triton X-114 extraction of *Leptospira*. *L. kirschneri* was extracted with 0.1% Triton X-114 by a modification of the method described previously (14). In brief, culture-attenuated *L. kirschneri* organisms were washed in phosphate-buffered saline–5 mM $MgCl_2$ and extracted in the presence of 0.1% protein grade Triton X-114 (Calbiochem), 10 mM Tris (pH 8), 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 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- and aqueous-phase proteins were precipitated with acetone.

Amino acid sequencing of an internal polypeptide fragment. LipL36 was obtained by treatment of *L. kirschneri* with Triton X-114 (see above). The Triton X-114 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 36-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, and the current was disconnected for 45 min, followed by completion of electrophoresis. The polypeptide fragments were subjected to SDS-PAGE, transferred to a Trans-Blot polyvinylidene difluoride protein sequencing membrane (Bio-Rad, Richmond, Calif.), and submitted to the University of California—Los Angeles (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 phenylthiohydantoin amino acids.

Southern blot analysis. *L. kirschneri* DNA was prepared by the method of Yelton and Charon (49). Genomic DNA from other leptospiral strains was kindly supplied by C. A. Bolin. 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 (35). Filters were prehybridized for 3 h at 37°C in buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 0.05% sodium PP_i, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml. The filters were then hybridized overnight at 37°C with radiolabeled probe.

A degenerate oligonucleotide probe of 23 bp was synthesized based on the N-terminal amino acid sequence of the LipL36 proteolytic fragment. Synthetic oligonucleotides were prepared with an automated oligonucleotide synthesizer (380B; Applied Biosystems, Inc.). The filters were washed at 47°C in a solution containing 3.0 M tetramethylammonium chloride (Aldrich), 50 mM Tris (pH 8.0), 2.0 mM EDTA, and 1.0% SDS, as previously described (47). The degenerate oligonucleotide probe was end labeled with [^{32}P]dATP by T4 polynucleotide kinase (Promega). Southern hybridization was also performed with a *lipL36* gene fragment probe labeled with [^{32}P]dCTP by the random priming method (U.S. Biochemicals). For the *lipL36* gene fragment probe, the filters were washed in 2× SSC at 40°C.

Cloning and sequencing of the *lipL36* gene. Standard recombinant DNA procedures were performed as described elsewhere (35). Restriction endonuclease digests were performed as recommended by the suppliers (New England Biolabs and Promega). *Eco*RI fragments of *L. kirschneri* genomic DNA were ligated into the Lambda-Zap II vector (Stratagene). The ligated DNA was packaged with Gigapack II Gold packaging extract (Stratagene) and stored in 0.3% chloroform at 4°C. The plaque titer was determined by infecting *E. coli* PLK F′ (Stratagene). Plaques were plated, transferred to filters in duplicate, and processed as previously described (35). The oligonucleotide probe hybridization and washing conditions described above for Southern hybridization were used. Recombinant pBluescript SK(−) clones were recovered from phage producing positive plaques by in vivo excision according to the manufacturer's recommendations. 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 with the DNA Strider program (23). Homology searches were performed with the BLAST, FASTA, and Profile Search programs, which are found in the University of Wisconsin Genetics Computer Group (GCG), Inc., package, version 7.0 (12). Secondary-structure predictions were based on analysis with the programs PEPPLOT and PLOTSTRUCTURE, which are also found in the GCG package.

Antisera. Antiserum to leptospiral GroEL was a generous gift of B. Adler (Monash University, Clayton, Victoria, Australia). Murine monoclonal antibody F71C2 against serovar grippotyphosa (16) was a generous gift of Rudy Hart-

skeerl (Royal Tropical Institute, Amsterdam, The Netherlands). Antisera to OmpL1 and LipL41 were prepared as previously described (13, 38). Briefly, New Zealand White rabbits were immunized with purified His6 fusion proteins expressed by *E. coli* JM109 (Invitrogen) that had been transformed with the pRSET plasmid (Invitrogen) containing either the *ompL1* or the *lipL41* gene (13, 38).

Antiserum to LipL36 was prepared as follows. Since there was not a convenient restriction endonuclease site near the amino terminus of the mature LipL36 protein, PCR was used to amplify the portion of the *lipL36* gene encoding the mature protein, beginning with the first residue after the amino-terminal cysteine. The 5′ oligonucleotide contained the nucleotide sequence coding for the 6 amino acids following the amino-terminal cysteine of mature LipL36, including a *Bgl*II restriction endonuclease site (underlined): 5′-TTA ACG AGA TCT AAA AGT GAC GAC GAT GAT-3′. The 3′ oligonucleotide consisted of a 24-bp nucleotide sequence beginning 133 bp downstream of the *LipL36* stop codon: 5′-CAT GAT AAA AAT TGA AAA TGA TTC AAG AAT-3′. The nucleotide sequence between the *LipL36* stop codon and the 3′ oligonucleotide sequence includes a unique *Hin*dIII restriction endonuclease site. *L. kirschneri* genomic DNA was used as the template. The 1,144-bp *Bgl*II-*Hin*dIII fragment of the amplified *lipL36* gene was ligated into pRSETb (Invitrogen) digested with *Bgl*II and *Hin*dIII. The resulting construct, pRSETb-JR2, was transformed into *E. coli* JM109. Expression of the His6-LipL36 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 His6-LipL36 fusion protein was solubilized in 6 M guanidine, purified by affinity chromatography using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen), and dialyzed in 20 mM Tris (pH 8)–50 mM NaCl–10% glycerol. Roughly 30 μg of His6-LipL36 was mixed with Freund's complete adjuvant and inoculated subcutaneously and intramuscularly into a New Zealand White male rabbit. The secondary immunization used roughly 30 μg of purified His6-LipL36 fusion protein in Freund's incomplete adjuvant. The rabbit was bled 2 weeks after the secondary immunization.

[^{3}H]palmitate radiolabeling and immunoprecipitation of native LipL36. A 35-ml culture containing 5×10^7 *L. kirschneri* organisms in the log phase of growth/ml 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 10^9/ml. Organisms were washed in 5 mM $MgCl_2$ in PBS. A sample for immunoprecipitation containing 8×10^9 *L. kirschneri* organisms was resuspended in 1.25 ml of a solution containing 10 mM Tris HCl (pH 8.0), 10 mM EDTA, and 1 mM phenylmethysulfonyl fluoride. To this suspension was added 12.5 μl of 10% protein grade Triton X-100 (Calbiochem), followed by gentle agitation 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 (at 56°C for 30 min) LipL36 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 LipL36 in *E. coli*. PCR was used to amplify the *lipL36* gene. The 5′ oligonucleotide contained an *Nco*I restriction endonuclease site (underlined), followed by nucleotides 3 to 20 of the *lipL36* gene: 5′-GT TCT TCC ATG GGG AGA AGA AAC ATA ATG AA-3′. The 3′ oligonucleotide contained an *Xho*I restriction endonuclease site (underlined), followed by the last 18 nucleotides of the *lipL36* gene (including the TAA stop codon) in antiparallel: 5′-TTC TAA CTC GAG TTA GTA TCT AGG ATA AGT-3′. *L. kirschneri* genomic DNA was used as the template. The 1,144-bp amplified *lipL36* gene was digested with *Nco*I and *Xho*I and ligated into pET15b (Novagen) digested with *Nco*I and *Xho*I. The resulting construct, pET15b-LipL36, was transformed into *E. coli* JM109(DE3) (Promega). Expression of LipL36 was achieved by IPTG induction. After induction, samples were separated by SDS-PAGE and probed with LipL36 antiserum. Processing was inhibited by the addition of globomycin (dissolved in ethanol) at a final concentration of 200 μg/ml immediately prior to the addition of IPTG. The final ethanol concentration was 2%; control experiments without globomycin used ethanol at the same concentration. Globomycin was a generous gift of M. Inukai (Sankyo Company, Tokyo, Japan).

***L. kirschneri* infection of hamsters.** Two groups of Golden Syrian hamsters, consisting of approximately equal numbers of males and females, were infected with *L. kirschneri* RM52. The first group of hamsters consisted of 21 5-week-old pups inoculated intraperitoneally (i.p.) with serial 10-fold dilutions of virulent *L. kirschneri* grown in liquid culture. Ten days later, liver tissue from one of the hamsters in the first group was used as a source of host-adapted organisms. The infected liver tissue was divided and incubated for 5 min in 100% normal rabbit serum. The rabbit serum containing host-adapted *L. kirschneri* was examined by dark-field microscopy, and 0.3 ml of this material was inoculated into a second group of hamsters (four 6-month-old female adults and nine 7-week-old pups). Hamsters from either group surviving 28 days after challenge were euthanized, and serum was harvested for testing in the LipL36 enzyme-linked immunosorbent assay (ELISA).

LipL36 ELISA. Immulon microtiter plates (Dynatech) were coated at 37°C overnight with 125 ng of His6-LipL36 fusion protein in 0.05 M sodium carbonate buffer (pH 9.6). The plates were washed three times with PBS containing 0.05% Tween 20, followed by the addition of 200 μl of blocking buffer (PBS containing 0.05% Tween 20 and 1% nonfat dried milk), and were incubated at 37°C for 1 h. After removal of the blocking buffer, 100 μl of antisera diluted 1:100 with blocking buffer was added, and the plates were incubated at 37°C for 2 h. After three washes, 100 μl of 1:2,000-diluted mouse monoclonal anti-hamster immunoglobulins (Sigma) was added, and the plates were incubated at 37°C for 2 h. After three more washes, 100 μl of 1:5,000-diluted sheep anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham) was added, and the plates were incubated at 37°C for 2 h. After three more washes, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was added, and the plates were incubated at 37°C for 20 min on an orbital shaker. Then 100 μl of a 1 M solution of sulfuric acid was added to stop the reaction. Absorbance was read at a wavelength of 450 nm with a microplate reader (model 550; Bio-Rad). Each serum sample was tested four separate times. Absorbance readings were normalized by subtracting the value obtained from antigen-negative control wells. Statistical analysis was performed by using Student's *t* test for two independent means.

Nucleotide sequence accession number. The nucleotide sequence of the *lipL36* gene from *L. kirschneri* RM52 has been deposited in the GenBank database under accession no. AF024626.

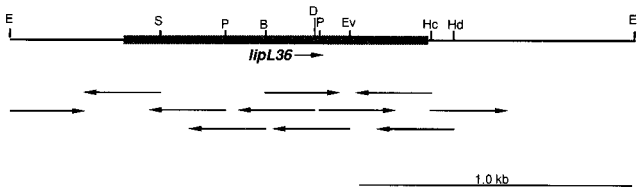

FIG. 1. Partial restriction map of the 2.3-kb *Eco*RI fragment containing the *lipL36* gene and strategy for determining the nucleotide sequence. The *lipL36* gene is 1,092 bp; its location is indicated by the shaded region. The arrows below the map indicate the direction and extent of sequence analysis. Letters above the map represent the following restriction enzymes: *Eco*RI (E), *Bam*HI (B), *Dra*I (D), *Eco*RV (Ev), *Hin*dIII (Hd), *Hin*cII (Hc), *Pvu*II (P), and *Sau*3A (S).

RESULTS

Design of oligonucleotide probes and cloning of the *lipL36* gene. Staphylococcal V8 protease digestion of LipL36 resulted in fragments with molecular masses of 21, 9, and 5 kDa. N-terminal amino acid sequence analysis of the 21-kDa fragment revealed the sequence YFGKTVLVRPSEQAKQKQIVLL. A 23-bp oligonucleotide probe with 256-fold degeneracy, GARC ARGCNAARCARAARCARAT, was designed based on the portion of sequence EQAKQKQI. The oligonucleotide probe independently identified a 2.3-kb *Eco*RI fragment by Southern hybridization of the *L. kirschneri* genome. The 2.3-kb *Eco*RI fragment was cloned from a partial Lambda-ZAP II (Stratagene) library of *L. kirschneri* genomic DNA as described previously (13).

Sequence analysis of the *lipL36* gene. Restriction mapping, Southern blot analysis, and DNA sequencing revealed that the entire *lipL36* gene is encoded by the 2.3-kb *Eco*RI fragment (Fig. 1). An intact open reading frame was identified 430 bp downstream from the *Eco*RI site. The *lipL36* structural gene consists of 1,092 bases encoding a protein of 364 amino acids. The locations of *E. coli*-like −35 (TTGACC) and −10 (TAT TAT) promoter regions are shown in Fig. 2. A consensus ribosome-binding site (AAGAGG) is also present upstream of the initiation codon. Between the promoter and the ribosome-binding site there is an inverted repeat which may function as an operator. Another inverted repeat, which may function as a rho-independent transcription terminator, is found 30 bp downstream from the termination codon (Fig. 2).

As expected for a lipoprotein, the deduced amino acid sequence begins with a 20-residue signal peptide, represented by the N-terminal peak on the hydrophobicity plot (data not shown). The LipL36 sequence conforms to the rules established for prokaryotic lipoprotein signal peptides (15, 28). The LipL36 signal peptide has a basic amino-terminal region (in-

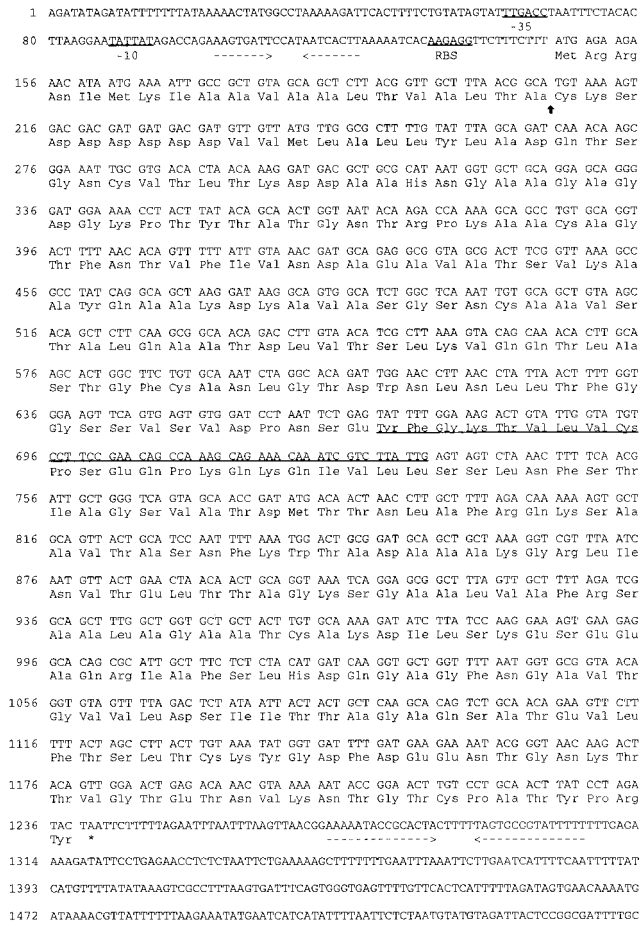

FIG. 2. Nucleotide sequence and deduced amino acid sequence of *lipL36*. Putative sigma 70 −35 and −10 promoter regions and ribosome-binding site (RBS) are shown. The putative lipoprotein signal peptidase cleavage site is indicated by an arrow. The amino acid sequence obtained from the staphylococcal V8 protease digestion of the native protein is underlined. Asterisk, location of the TAA stop codon. Horizontal dashed arrows, inverted repeats. The inverted repeat downstream of the termination codon may function as a rho-independent transcription terminator.

cluding arginines at positions 2 and 3), a hydrophobic core (amino acids 8 through 20), and a carboxy-terminal Leu-X-Y-Cys signal peptidase II cleavage site. The hydrophobicity of the signal peptide core region is reflected by the broad N-terminal peak of 2.8 on the Kyte-Doolittle hydrophobicity plot (data not shown). After cleavage of the 20-amino-acid signal peptide by leptospiral signal peptidase II, the mature polypeptide would have a predicted molecular mass of 35.3 kDa. Staphylococcal V8 protease is known to cleave peptides following acidic amino acids. Immediately following the glutamic acid residue 174 is a sequence that is identical in 20 of 22 amino acids to the sequence obtained by N-terminal amino acid sequence analysis of the native protein (Fig. 2). Beginning on the fourth residue of the mature polypeptide sequence is an unusual cluster of six consecutive aspartate residues. As shown in Fig. 3, many spirochetal lipoproteins contain multiple acidic residues in this region. Database searching using the FASTA, BLAST, and Profile Search programs failed to reveal significant amino acid homologies.

Conservation of the *lipL36* gene in *Leptospira* spp. To address the frequency and distribution of the *lipL36* gene, we

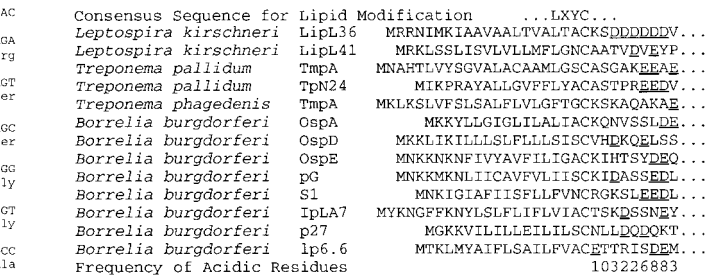

FIG. 3. Amino acid similarities between the amino-terminal sequences of LipL36 and 12 other spirochetal lipoproteins. The LipL36 sequence contains the L-X-Y-C consensus sequence for lipid modification. The amino terminus of LipL36 has a cluster of six consecutive aspartate residues located from position +4 to +9 of the mature polypeptide sequence. Acidic amino acids (underlined) occur commonly near the amino termini of the spirochetal outer membrane-associated lipoproteins listed here. This is especially true in the region from position +7 to +9, where 56% (22 of 39) of the residues are either aspartate or glutamate.

performed Southern hybridization analysis with a fragment of the *lipL36* gene. Figure 4 shows the results of probing genomic digests from representative strains from most of the known pathogenic and nonpathogenic *Leptospira* spp. The *lipL36* gene appeared to be present in nine strains, representing all six of the pathogenic *Leptospira* species tested. The only two pathogenic strains that appeared not to contain the *lipL36* gene were members of the species *L. kirschneri*. This is surprising, considering that the *lipL36* gene was isolated from *L.*

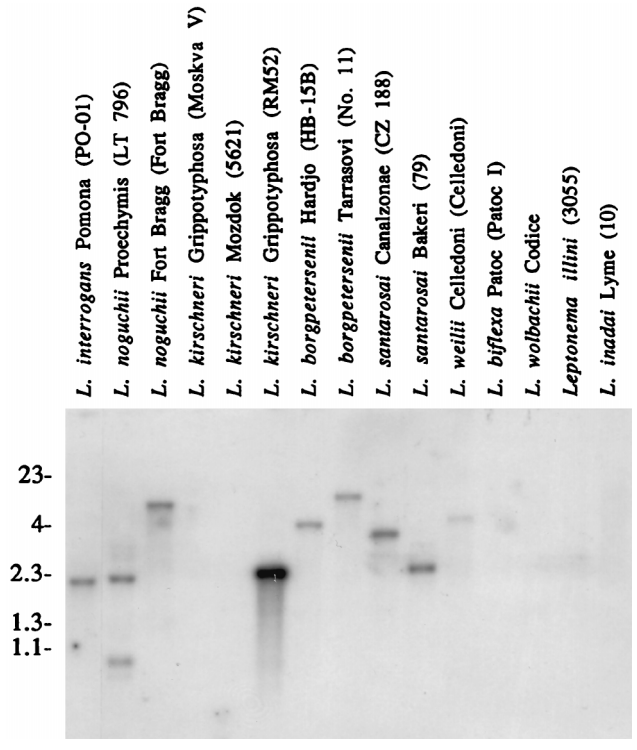

FIG. 4. Southern blot analysis of *Eco*RI-restricted genomic DNA from selected strains of *Leptospira* spp. A fragment of the *lipL36* gene was used to probe *Eco*RI genomic digests of 11 pathogenic and 4 nonpathogenic leptospiral strains. The *lipL36* gene was present in all six tested pathogenic *Leptospira* species, and in all tested strains of these species except for two of three *L. kirschneri* strains. The gene was not present in nonpathogenic strains of *Leptospira*. The locations of DNA size standards (in kilobases) are indicated on the left.

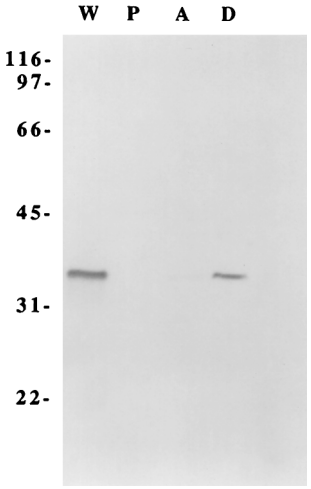

FIG. 5. Behavior of LipL36 in Triton X-114 and specificity of LipL36 antiserum. Triton X-114 fractions of *L. kirschneri* organisms were separated by SDS-PAGE and probed with LipL36 antiserum. Fractions analyzed were the whole organism (W) and Triton X-114-insoluble pellet (P), and aqueous-phase (A) and detergent-phase (D) material. The number of cell equivalents was the same in all four lanes. Locations of molecular size standards are shown (in kilodaltons) on the left. LipL36 is completely solubilized by Triton X-114 and partitions exclusively into the detergent phase.

kirschneri serovar grippotyphosa strain RM52. *Leptospira* species assignments are based on DNA-DNA hybridization studies (34). This is consistent with the earlier finding that Southern blots with the *ompL1* probe of *L. kirschneri* RM52 show better binding to other *L. kirschneri* strains than to other leptospiral species (13). For these reasons, it is likely that the lack of binding of the *lipL36* probe represents absence of the gene rather than poor hybridization. The *lipL36* gene appears to be present in a single copy; the only strain in which more than one band was present was *L. noguchii* serovar Proechymis (LT 796), a finding which could be explained by an *Eco*RI site within the hybridization region. The gene was not detected in *L. biflexa*, *L. wolbachii*, or *L. inadai*, three nonpathogenic *Leptospira* species, or in the related nonpathogen *Leptonema illini.*

Behavior of LipL36 during Triton X-114 extraction and phase partitioning. We analyzed the behavior of LipL36 in the nonionic detergent Triton X-114 and tested the specificity of the LipL36 antiserum. Triton X-114 extraction of *L. kirschneri* solubilizes the leptospiral outer membrane, including the LPS, the porin OmpL1, and the surface-exposed lipoprotein LipL41 (13, 14, 38). LipL36 antiserum was used to probe Triton X-114 fractions of *L. kirschneri*, revealing a single band in both the whole-organism and the detergent phase. No material was detected in the aqueous phase, indicating selective partitioning of LipL36 into the Triton X-114 detergent phase (Fig. 5). Lipoproteins characteristically partition into the Triton X-114 detergent phase because of the hydrophobicity of the fatty acids. Unlike LipL41 (38), LipL36 was completely extracted in 0.1% Triton X-114, as demonstrated by complete removal from the detergent-insoluble pellet (Fig. 5).

***L. kirschneri* acylates LipL36.** Intrinsic labeling of culture-attenuated *L. kirschneri* with [^{3}H]palmitate resulted in the incorporation of label in leptospiral LPS, which appears diffusely at the bottom of the whole-organism lane in Fig. 6, as well as in at least 10 proteins which form discrete bands in the whole-organism lane. Immunoprecipitation experiments with anti-LipL36 antiserum (Fig. 6) confirm that LipL36 is the second-smallest lipoprotein identified in this autoradiograph.

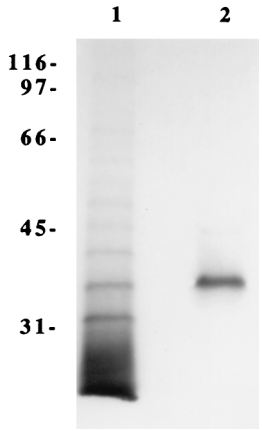

FIG. 6. LipL36 is selectively acylated by *L. kirschneri*. An autoradiogram of *L. kirschneri* proteins intrinsically labeled by [^{3}H]palmitate and separated by SDS-PAGE is shown. Lane 1, total *L. kirschneri* proteins; lane 2, material immunoprecipitated by addition of LipL36 antiserum to a Triton X-100 extract of *L. kirschneri*. Locations of molecular size standards are shown (in kilodaltons) on the left.

Globomycin inhibits processing of LipL36. In order to demonstrate that LipL36 is processed by lipoprotein signal peptidase, *E. coli* JM109 containing pET-15b-LipL36 was treated with IPTG with or without globomycin, a selective inhibitor of lipoprotein signal peptidase. The majority of LipL36 expressed was unprocessed, with an apparent molecular mass about 2 kDa greater than that of the processed form (Fig. 7). The apparent molecular mass of the processed form of LipL36 was identical to that of the native protein (data not shown). Induction in the presence of globomycin inhibited processing of LipL36, resulting in a decrease of the processed form. These data indicate that the LipL36 signal peptide is processed by *E. coli* lipoprotein signal peptidase. The observation that processing was incomplete suggests that the LipL36 signal peptide is processed more efficiently in *L. kirschneri* than in *E. coli*, a finding also made in the case of LipL41 (38).

Complete solubilization of LipL36 and LPS in 0.1% Triton X-100. *Leptospira* species produce a glycolipid LPS that is similar in structure to gram-negative LPS. By virtue of its surface exposure and by analogy with gram-negative bacteria, we concluded that leptospiral LPS is a useful marker for the outer membrane. We have shown previously, using periodate silver stain, that leptospiral LPS solubilization is essentially

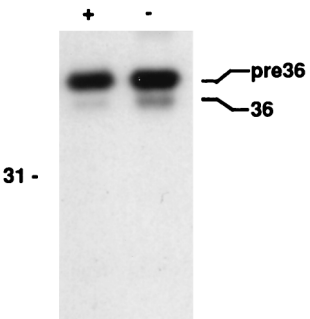

FIG. 7. Globomycin inhibition of LipL36 processing in *E. coli. E. coli* JM109(DE3) containing pET15b-LipL36 was treated with 0.1 mM IPTG with (+) or without (−) globomycin and was probed with LipL36 antiserum. The locations of unprocessed (pre36) and processed (36) LipL36 are shown on the right. The location of a molecular size standard is shown (in kilodaltons) on the left.

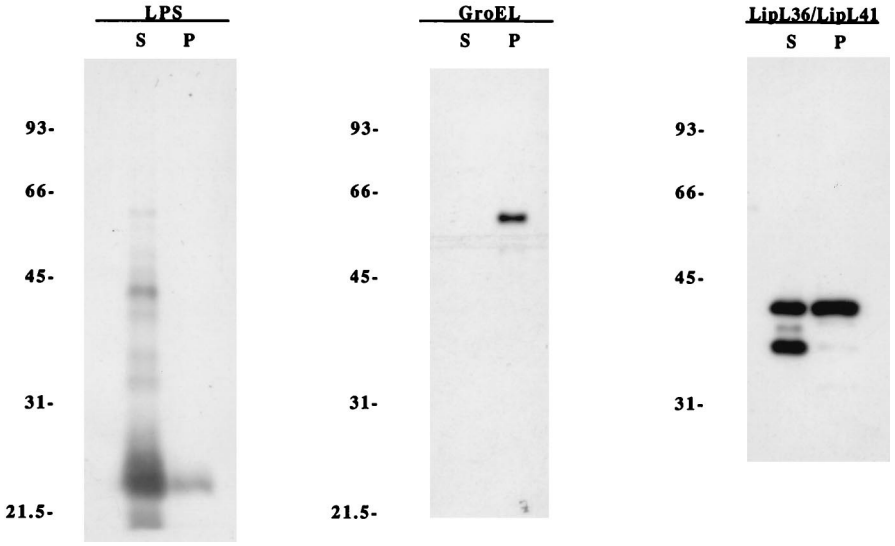

FIG. 8. Fate of *L. kirschneri* antigens after extraction with 0.1% Triton X-100. *L. kirschneri* antigens in the Triton X-100-soluble fraction (S) or insoluble pellet (P) were separated by SDS-PAGE and visualized by probing with a monoclonal antibody specific for serovar grippotyphosa LPS (left panel), with antisera to leptospiral GroEL (middle panel), or with a mixture of antisera to LipL36 and LipL41 (right panel). Locations of molecular size standards are shown (in kilodaltons).

complete in 0.1% Triton X-114 (14). In the present report we show that 0.1% Triton X-100 has a similar pattern of LPS solubilization by probing an immunoblot with the LPS monoclonal antibody (Fig. 8, left panel). As expected, the protoplasmic cylinder marker GroEL was found to remain with the Triton X-100-insoluble fraction (Fig. 8, middle panel). Like LPS, LipL36 is completely solubilized by Triton X-100 (Fig. 8, right panel). In contrast, at least half the LipL41 was found to be insoluble in Triton X-100 (Fig. 8, right panel). These differences in Triton X-100 solubility may indicate that LipL36 is localized exclusively in the outer membrane, while LipL41 is present in both the inner and outer membranes.

Changes in LipL36 levels during growth in culture. Initial studies revealed that leptospiral membrane protein preparations differed in LipL36 representation. For this reason, we explored the possibility that LipL36 levels were affected by the growth phase of the culture from which the membrane proteins were prepared. Whole-cell samples were obtained at various times after inoculation of culture medium with *L. kirschneri*. Immunoblot analysis of these samples with LipL36 and LipL41 antisera indicated that LipL41 content remained constant, while LipL36 content decreased markedly as cell density increased (Fig. 9). These growth phase-dependent changes in LipL36 expression were found to be independent of passage number (data not shown).

Humoral immune response to leptospiral proteins during infection with virulent *L. kirschneri*. We used the hamster model of leptospirosis to compare the humoral immune response to infection with culture-adapted versus host-adapted *L. kirschneri*. Hamsters are extremely sensitive to infection with virulent *Leptospira* species. In the first group, only 4 of 21 (19%) hamsters survived i.p. challenge with virulent *L. kirschneri* grown in culture. In the second group, three of four adults and one of nine 7-week-old hamsters survived to day 28 after infection with host-adapted *L. kirschneri*. An attempt was made to determine the concentration of host-adapted organisms by dark-field microscopy. No organisms were seen in 10 high-power fields, indicating that the concentration of host-adapted organisms was below the sensitivity of dark-field microscopy ($<10^5$/ml). Sera from all survivors in both groups were tested by LipL36 ELISA. The mean LipL36 ELISA reading of sera from animals surviving challenge with culture-adapted *L. kirschneri* (0.575) was significantly higher ($P < 0.001$) than that of sera from animals surviving challenge with host-adapted *L. kirschneri* (0.320).

DISCUSSION

LipL36 is a 36-kDa leptospiral outer membrane lipoprotein. Several lines of evidence support the conclusion that LipL36 is lipid modified at its amino-terminal cysteine residue. First of all, LipL36 was found to be blocked to N-terminal amino acid sequencing until subjected to staphylococcal V8 protease digestion. Analysis of its deduced amino acid sequence reveals a signal peptide followed by an L-X-Y-C consensus lipoprotein signal peptidase cleavage site, a pattern homologous to that of known bacterial lipoproteins (43). LipL36 is labeled by [9,10(*n*)-^{3}H]palmitate intrinsic labeling of *L. kirschneri*. Like other spirochetal lipoproteins, LipL36 selectively partitions into the Triton X-114 hydrophobic, detergent phase. Finally, processing of LipL36 is inhibitable by globomycin, a selective inhibitor of lipoprotein signal peptidase. The combination of the sequence information, palmitate labeling, and globomycin inhibition satisfies three of Wu's criteria for definition of a lipoprotein (15). Although *Leptospira* species metabolize fatty acids by beta-oxidation, intrinsic labeling of *L. kirschneri* with [9,10(*n*)-^{3}H]palmitate appeared to selectively label LPS and several other proteins, including LipL36 (Fig. 6) and LipL41 (38). It should also be noted that, as we have discussed previously, incorporation of the tritium label of [9,10(*n*)-^{3}H]palmitate into amino acids would be extremely inefficient relative to modification of lipoproteins by one or more molecules of [9,10(*n*)-^{3}H]palmitate (38).

Very little is known about secretory pathways in spirochetes. Comparison of sequences and membrane destination of lipoproteins such as LipL36 and LipL41 may provide insights into how leptospiral membrane proteins are localized. In this regard, there are several unusual, and potentially relevant, features of the deduced amino acid sequence of LipL36. The first is a series of six consecutive aspartate residues located

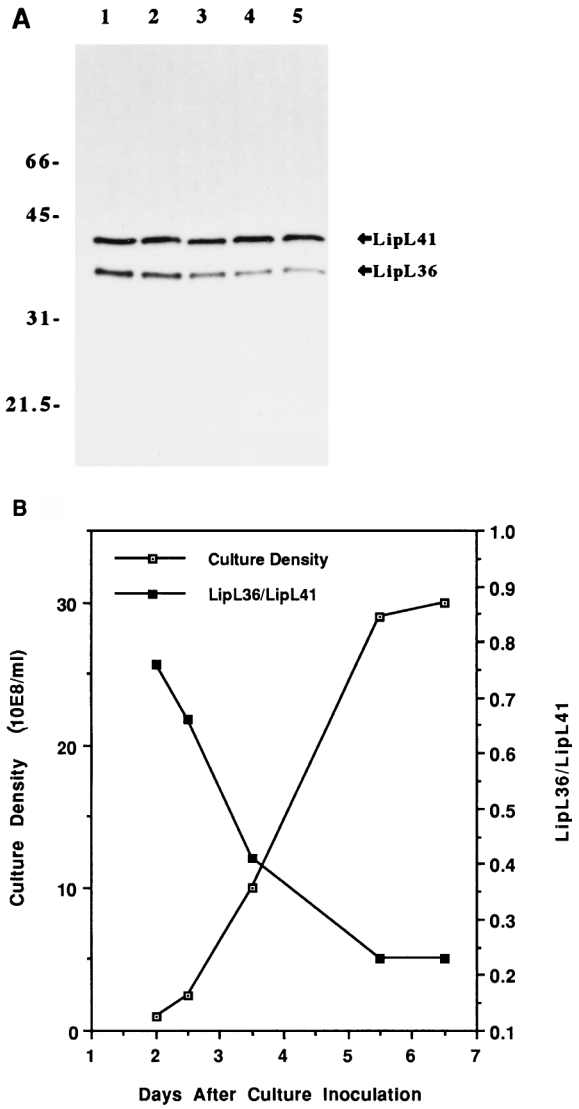

FIG. 9. Changes in LipL36 levels during growth in culture. (A) Immunoblot of *L. kirschneri* proteins separated by SDS-PAGE and probed with antisera to LipL36 and LipL41. Each lane contains 2.5×10^7 *L. kirschneri* cells. Cells were obtained 2.0 days (lane 1), 2.5 days (lane 2), 3.5 days (lane 3), 5.5 days (lane 4), and 6.5 days, (lane 5) after inoculation of the culture. Locations of molecular size standards are shown (in kilodaltons) on the left. (B) Plot of the time after culture inoculation versus the cell density and ratio of LipL36 levels to LipL41 levels as determined by densitometric analysis of panel A. While LipL41 levels remain constant during growth of cultivated *L. kirschneri*, LipL36 levels are highest during early-log phase.

from position +4 to +9 of the mature polypeptide sequence. The hydrophilicity of the aspartate cluster is reflected by a deep negative inflection in the Kyte-Doolittle plot occurring immediately after the signal peptide peak (data not shown). Figure 3 demonstrates that acidic amino acids occur commonly near the amino terminus of spirochetal outer membrane-associated lipoproteins. Acidic residues in this region may be important in guiding spirochetal lipoproteins to the correct secretory pathway (28). Substitution of serine for aspartate near the amino terminus of the *E. coli* murein lipoprotein alters outer membrane export efficiency (48). Experimental confirmation that the amino-terminal region of lipoproteins contains secretory information will require the development of tools for genetic manipulation of these bacteria.

Another unusual feature of the LipL36 sequence is the abundance of alanine residues. Almost 16% (55 of 344) of the residues in the mature LipL36 protein are alanines, and 25 of these alanine residues are arranged in pairs or triplets. Experiments involving model polypeptides have shown that alanine-containing polypeptides form unusually stable alpha-helices (25). Roughly 38% of the LipL36 sequence is predicted to be alpha-helical by Chou-Fasman analysis, and 30% (39 of 131) of the residues in the alpha-helical regions are alanines. The TmpB proteins of *Treponema phagedenis* and *Treponema pallidum* are rich in alanine (50). Several lipoproteins of pathogenic *Neisseria* species have also been found to be rich in alanine (9a, 16a, 17). The alpha-helical conformation of LipL36 could be stabilized by the presence of 14 potential salt bridges conforming to the N + 4 rule (24).

Previous studies have suggested that the nonionic Triton detergents selectively solubilize the leptospiral outer membrane (14, 51). Although LipL36 and LipL41 are both outer membrane lipoproteins, they differ in their pattern of Triton X-100 solubilization (Fig. 8), indicating that LipL36 and LipL41 differ in their outer/cytoplasmic (inner) membrane distribution. Controls used in this experiment were LPS, an outer membrane marker, and GroEL, a cytoplasmic cylinder marker. Like LPS, LipL36 was completely solubilized by 0.1% Triton X-100, implying that LipL36 is found exclusively in the outer membrane. In contrast, LipL41 appears to be located in both leptospiral membranes, a pattern of distribution found for other spirochetal lipoproteins (5, 33, 39). Previous surface immunoprecipitation studies indicate that LipL41 is surface exposed, while LipL36 is not (38). In combination, these data suggest that LipL36 is efficiently exported to the periplasmic leaflet of the outer membrane but is unable to gain access to the outer leaflet of the outer membrane. While LipL41 does contain the signal(s) required for export to the leptospiral surface, its export to the outer membrane appears to be incomplete. An alternative interpretation of the findings presented in Fig. 8 is that differences in Triton X-100 solubility are innate properties of LipL36 and LipL41 that do not reflect cellular localization. Ultimately, ultrastructural studies and improved outer membrane isolation techniques will be necessary to more firmly establish the membrane distribution of leptospiral antigens.

The evidence presented here indicates that LipL36 is differentially expressed during growth in culture. LipL36 is present in large amounts in cultivated *L. kirschneri* during early-log-phase growth but is found to decrease beginning in mid-log phase (Fig. 9). One interpretation of these data is that LipL36 expression is downregulated during late-log-phase growth. Another possible interpretation is that LipL36 is subject to digestion by an endogenous protease and that this process becomes more active during late-log-phase growth. However, proteolytic digestion of LipL36 is unlikely to explain the changes in LipL36 levels for the following reasons: (i) organisms isolated throughout this experiment retained gross structural integrity and >99% motility, (ii) samples were handled at 4°C and denatured in FSB immediately after isolation, and (iii) other than changes in the LipL36 level, SDS-PAGE protein profiles of the samples analyzed in Fig. 9 were essentially identical (data not shown). We did not examine the effects of cell density on LipL36 expression. Cell-density-dependent expression of the *B. burgdorferi* outer membrane lipoprotein P35 has been described previously (18). However, in contrast to LipL36, P35 expression is upregulated when *B. burgdorferi* enters the stationary phase of growth in culture.

A universal property of pathogenic spirochetes is the ability to cause chronic infections. One proposed mechanism by which spirochetes are able to persist in the host is evasion of the host immune response by downregulation of OMP expression (4, 14, 20, 29, 32, 44, 45). Analysis of the humoral immune response to LipL36 during *L. kirschneri* infection suggests that LipL36 expression is downregulated during mammalian infection. The antibody response to LipL36 was significantly stronger in hamsters infected with culture-adapted *L. kirschneri* than in those infected with host-adapted *L. kirschneri*. We acknowledge that measurement of antibody levels is an indirect approach to studying in vivo OMP expression. Alternative interpretations of these data could involve effects of challenge inoculum and hamster age on the humoral immune response. However, downregulation of LipL36 expression in vivo has recently been confirmed by more direct evidence involving immunohistochemistry (2). Based on these findings, LipL36 should be a useful tool for studying the adaptation of pathogenic *Leptospira* species to the host environment. Northern blot studies are needed to determine whether or not regulation of LipL36 expression occurs at the transcriptional level. It may be possible to apply molecular strategies for identification of LipL36 regulatory proteins, a key step towards an understanding of the mechanisms by which pathogenic *Leptospira* species control their response to the diverse environments encountered during their life cycle.

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