Surfaceome of *Leptospira* spp.

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The identification of the subset of outer membrane proteins exposed on the surface of a bacterial cell (the surfaceome) is critical to understanding the interactions of bacteria with their environments and greatly narrows the search for protective antigens of extracellular pathogens. The surfaceome of *Leptospira* was investigated by biotin labeling of viable leptospires, affinity capture of the biotinylated proteins, two-dimensional gel electrophoresis, and mass spectrometry (MS). The leptospiral surfaceome was found to be predominantly made up of a small number of already characterized proteins, being in order of relative abundance on the cell surface: LipL32 > LipL21 > LipL41. Of these proteins, only LipL32 had not been previously identified as surface exposed. LipL32 surface exposure was subsequently verified by three independent approaches: surface immunofluorescence, whole-cell enzyme-linked immunosorbent assay (ELISA), and immunoelectron microscopy. Three other proteins, Q8F8Q0 (a putative transmembrane outer membrane protein) and two proteins of 20 kDa and 55 kDa that could not be identified by MS, one of which demonstrated a high degree of labeling potentially representing an additional, as-yet-uncharacterized, surface-exposed protein. Minor labeling of p31$_{LipL45}$, GroEL, and FlaB1 was also observed. Expression of the surfaceome constituents remained unchanged under a range of conditions investigated, including temperature and the presence of serum or urine. Immunization of mice with affinity-captured surface components stimulated the production of antibodies that bound surface proteins from heterologous leptospiral strains. The surfaceomics approach is particularly amenable to protein expression profiling using small amounts of sample ($<10^7$ cells) offering the potential to analyze bacterial surface expression during infection.

Leptospirosis is a zoonosis of global distribution caused by infection with one of more than 230 serovars belonging to pathogenic species of *Leptospira* (10a, 25). Immunity to infection is mediated principally by antibodies, which opsonize leptospires for phagocytosis by both neutrophils and macrophages (29, 39) and also mediate complement-dependent killing (1). Lipopolysaccharide (LPS) is the major component of the leptospiral cell surface (10a, 41). It is the target antigen for antibodies that are agglutinating, opsonic, and protective (3, 11, 23, 24). However, LPS-mediated immunity is restricted to serovars which are antigenically related.

The leptospiral outer membrane contains few integral transmembrane proteins, with the trimeric porin OmpL1 being the only such protein that has been identified and characterized (14, 37). However, the membrane contains numerous lipoproteins, which are anchored to the membrane through their N-terminal lipid moieties (9, 13). Some of these have been shown to stimulate partial immunity in animal models. LipL32 delivered by recombinant adenovirus partially protected gerbils

from acute infection (6), while LipL41 showed synergistic immunoprotection with OmpL1; neither protein was protective when administered alone (18). Another outer membrane lipoprotein, LipL36, was shown to be expressed by leptospires growing in vitro but not within the mammalian host (4). Although only partial protection has been achieved to date, leptospiral outer membrane proteins constitute attractive vaccine candidates because they are well conserved across the pathogenic species of *Leptospira* (9, 13).

Clearly, the leptospiral surface is important when we consider the interaction of bacteria with host cells and tissues in the context of pathogenesis and immunity to infection. However, to date there have been no global studies undertaken to identify the components of the outer membrane that are exposed on the leptospiral cell surface. Such studies are critical because they reduce the number of proteins that need to be assessed as potential vaccine targets and highlight proteins that are likely to be involved directly in interactions with the host. For example, LipL36 was shown to be anchored to the inner leaflet of the outer membrane and therefore to be localized to the periplasm (13, 38, 41). Due to the different techniques utilized to assess surface exposure, there is no information regarding the relative exposure of the different leptospiral surface proteins, which is also of critical importance when proteins are selected to assess as potential vaccinogens.

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The aim of the present study was therefore to identify all of the protein components of the leptospiral surface by labeling of viable leptospires, affinity capture of the labeled proteins, and their identification by mass spectrometry (MS). This process also allowed the relative surface exposure of leptospiral surface components to be approximated. In addition, several independent experiments were undertaken to verify the surface exposure of the major outer membrane protein (MOMP) LipL32.

MATERIALS AND METHODS

Growth of leptospires. Unless otherwise stated the following strains of *Leptospira* were grown in EMJH medium at 30°C (22) and enumerated as described previously (2): *Leptospira borgpetersenii* serovar Hardjobovis strain LT1085, *L. borgpetersenii* serovar Tarassovi strain Perepelicin, *L. fainei* serovar Hurstbridge strain BUT6, *L. interrogans* serovar Australis strain Ballico, *L. interrogans* serovar Canicola strain Hond Utrecht IV, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Copenhageni strain M20, *L. interrogans* serovar Lai strain 56601, *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Zanoni strain Zanoni, *L. kirschneri* serovar Grippotyphosa strain Moskva V, and *L. kirschneri* serovar Grippotyphosa strain RM52. For growth-phase analysis, 5 ml of medium was inoculated with 2.5×10^6 leptospires from a log-phase culture and harvested at densities of 1×10^7, 1×10^8, 5×10^8, and 1×10^9 cells/ml. To examine culture conditions, 5-ml cultures were allowed to reach a density of 3×10^7 cells/ml and were then shifted to 37°C or retained at 30°C, and 0.5 ml of either fresh medium, fetal bovine serum (CSL), or sterile-filtered mid-stream urine from a healthy donor was added. Subsequently, cultures were allowed to reach a final density of 5×10^8 cells/ml before surface labeling was performed. For the experiments used to verify LipL32 surface exposure, leptospiral strains were grown in EMJH medium supplemented with 1% fetal bovine serum.

Cell surface labeling. Culture volumes corresponding to 5×10^8 cells were centrifuged at $10,000 \times g$ to pellet cells. The leptospiral cell pellets were washed twice with phosphate-buffered saline (PBS; pH 7.2)–5 mM MgCl$_2$ and resuspended in the same buffer with the addition of 0.5 mg of sulfo-succinimidyl-6-(bitoinamido) hexanoate (Sulfo-NHS-LC Biotin; Pierce)/ml. The labeling reaction was allowed to proceed for 1 min before the residual Sulfo-NHS-LC-Biotin was quenched by the addition of Tris-HCl (pH 7.2) to a final concentration of 50 mM and incubation at room temperature for 10 min. Inactivated Sulfo-NHS-LC-Biotin was removed by two washes with PBS (pH 7.2)–5 mM MgCl$_2$. For the preparation of labeled sonicates, cells were lysed by three successive rounds of sonication (30 s, 20 kHz) and then labeled as described above. Finally, the cell pellets and sonicates were dried by using a vacuum centrifuge and stored for further analysis.

Affinity capture of labeled cell surface proteins. A cell pellet containing 10^{11} leptospires was resuspended in 9 ml of PBS (pH 7.2)–1% protein grade Triton X-100 (Calbiochem) and subjected to three successive rounds of sonication. A detergent extract was then obtained by collecting the supernatant after centrifugation at $12,000 \times g$. SoftLink resin (Promega) was preadsorbed and regenerated according to the manufacturer's instructions. A 1-ml bed volume of SoftLink resin equilibrated in PBS (pH 7.2)–1% Triton X-100 was combined with the 9 ml of detergent extract and incubated overnight at 4°C on a rotating wheel in the presence of 0.5% bacterial protease inhibitor cocktail (Sigma). The Soft-Link resin was then pelleted at $500 \times g$ and washed three times with PBS (pH 7.2)–1% protein grade Triton X-100. The resin was combined with 4 ml of PBS (pH 7.2)–1% protein grade Triton X-100-5 mM D-biotin (Sigma) and incubated overnight at 4°C on a rotating wheel in the presence of 0.5% bacterial protease inhibitor cocktail (Sigma catalog no. P8465). The SoftLink resin was pelleted at $500 \times g$, and the supernatant was collected. The supernatant was concentrated on an Amicon YM10 membrane (Millipore) and resuspended in distilled water. The affinity-captured proteins were precipitated with 10 volumes of acetone before resuspension in buffer for electrophoresis.

One-dimensional gel electrophoresis. Samples were solubilized in final sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, and 0.1% bromophenol blue in 20% glycerol and heated at 100°C for 5 min. Proteins were resolved by using a Mini-Protean II gel electrophoresis apparatus (Bio-Rad) utilizing a 12.5% polyacrylamide resolving gel and a 4% stacking gel.

Two-dimensional gel electrophoresis. Acetone-precipitated, affinity-captured surface proteins from 10^{11} leptospires were resuspended in 125 μl of membrane protein-specific sample solution (7 M urea, 2 M thiourea, 1% tetradecanoylamido-propyl-dimethyl amido-propane-sulfonate [ASB-14], 2 mM tributylphosphine, and 1% carrier ampholytes) by vortexing (31). Dried cell sonicates or cell pellets corresponding to 5×10^8 surface-labeled cells were resuspended in 500 μl of membrane protein-specific sample solution and treated with 150 U of *Serratia marcescens* endonuclease (Sigma). Material insoluble in the membrane protein-specific sample solution was removed by centrifugation at $12,000 \times g$. For the dried cell sonicates or cell pellets, 25 μl (2.5×10^7 cell equivalents) of the supernatants were combined with 100 μl of membrane protein-specific sample solution. The 125-μl samples were used to passively rehydrate 7-cm pH 4 to 7 and pH 3 to 10 NL immobilized pH gradient strips (Bio-Rad). Isoelectric focusing was performed by using a step-wise protocol with a final voltage of 3500V on a Multiphor II (Amersham) apparatus, equaling a final total of 10 kV · h. Focused immobilized pH gradient strips were reduced and alkylated for 30 min in a solution containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 1× Tris-HCl buffer (pH 8.8), 20% glycerol, 5 mM tributylphosphine, and 2.5% acrylamide monomer. The second dimension was performed by using a Bio-Rad Mini-Protean II gel electrophoresis apparatus (Bio-Rad) utilizing a 10% polyacrylamide gel. Proteins were visualized by sequential staining with Sypro Ruby (Molecular Probes) and Coomassie brilliant blue G-250 (Bio-Rad).

Biotin ligand blotting. For ligand blotting, 10^7 cell equivalents per lane were resolved by one-dimensional electrophoresis, and 2.5×10^7 cell equivalents per gel were resolved by two-dimensional electrophoresis. Proteins were transferred onto Immobilon-P membranes (Millipore) with a Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked overnight in 5% (wt/vol) skim milk buffer, washed twice in PBS containing 0.05% Tween 20 (PBS-T), and incubated in streptavidin-horseradish peroxidase conjugate (Amersham) at a dilution of 1:20,000 in PBS-T for 30 min. The membranes were then washed six times with PBS-T and developed with the ECL Western blot detection system (Amersham) before visualization with Hyperfilm (Amersham).

MALDI-TOF MS. Chains of protein spots were excised from gels by using a sterile scalpel and washed with 50 mM ammonium bicarbonate–100% acetonitrile (60:40 [vol/vol]). The gel pieces were dried in a vacuum centrifuge and rehydrated in 12 ng of sequencing-grade, modified trypsin (Promega)/μl for 1 h at 4°C. Excess trypsin solution was removed, and the rehydrated gel pieces were immersed in 50 mM ammonium bicarbonate and incubated overnight at 37°C. Eluted peptides were concentrated and desalted by using μ-C$_{18}$ Zip-Tips (Millipore) and washed with 10 μl of 5% formic acid. The bound peptides were eluted from the Zip-Tips in matrix solution (10 mg of α-cyano-4-hydroxycinnamic acid [Sigma]/ml in 70% acetonitrile) directly onto the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) target plate. MALDI-TOF mass spectra were acquired by using a PerSeptive Biosytems Voyager DE-STR equipped with a 337-nm nitrogen laser. All spectra were obtained in reflectron/delayed extraction mode, averaging 256 laser shots per sample. Two-point internal calibration of spectra was performed based upon internal porcine trypsin autolysis peptides (842.5 and 2,211.10 [M+H]$^+$ ions). A list of monoisotopic peaks corresponding to the mass of generated tryptic peptides was used to search the TrEMBL and SWISS-PROT databases via the PeptIdent program, while the Mascot program (33) was used to search the National Center for Biotechnology Information database. Successful identifications were based on the number of matching peptide masses and the percentage sequence coverage afforded by those matches.

Whole-cell ELISA. Flat-bottom polystyrene high-binding microtiter plates (Corning catalog number 9018) were coated with 100 μl per well with a 1:1,000 dilution of anti-Grippotyphosa monoclonal antibody F71C2 (21) in 0.05 M sodium carbonate (pH 9.6) at 37°C for 2 h. Plates were blocked with 200 μl of leptospiral culture medium for 2 h at 37°C. Portions (100 μl) of various concentrations of *L. kirschneri* serovar Grippotyphosa strain RM52, diluted in leptospiral culture medium, were added, followed by incubation at 30°C for 1 h. Wells were washed three times with 200 μl of leptospiral culture medium. Wells were incubated with 100 μl per well of primary antiserum at 30°C for 1 h, followed by three washes with 200 μl of leptospiral culture medium. Wells were incubated with 100 μl per well of a 1:1,000 dilution of horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin (Sigma) at 30°C for 1 h, followed by two washes with 200 μl of leptospiral culture medium and three washes with PBS. Enzyme-linked immunosorbent assay (ELISA) plates were developed by adding 100 μl per well of 0.01% (wt/vol) 3,3′,5,5′-tetramethylbenzidine in substrate buffer (0.03% [vol/vol] hydrogen peroxide, 25 mM citric acid, 50 mM Na$_2$HPO$_4$ [pH 5.0]) for 20 min in the dark at room temperature. The reaction was stopped by adding 25 μl of 2 N H$_2$SO$_4$, and the absorbance at 450 nm was measured.

Confocal microscopy. Confocal microscopy was performed with a Zeiss LSM 510 microscope. Slide chambers (Labtek) were coated with a 1:1,000 dilution of anti-Grippotyphosa monoclonal antibody F71C2 in PBS and then blocked with leptospiral culture medium. *L. kirschneri* cells were washed once in PBS, resuspended at a density of 2×10^9 per ml in PBS, and then incubated in the slide chamber for 1 h at 30°C. The slide chamber was washed twice with leptospiral culture medium. In experiments in which the bacteria were to be permeabilized, chambers were incubated in 1 ml of cold methanol for 10 min at −20°C, followed by two washes with leptospiral culture medium. Primary antisera diluted 1:100 in leptospiral culture medium were added. After incubation for 1 h at 30°C, the chambers were washed twice with leptospiral culture medium. Subsequently, the slides were incubated for 1 h in the dark at 30°C with Alexa-conjugated mouse anti-rabbit immunoglobulins (Molecular Probes) diluted 1:1,000 in leptospiral culture medium and washed twice with leptospiral culture medium. Organisms were counterstained with a 1:1,000 dilution of Syto 83 (Molecular Probes) in leptospiral culture medium overnight in the dark at room temperature. Chambers were washed twice with leptospiral culture medium and twice with water. Mounting medium was added, and a coverslip was sealed with acrylic. Staining was visualized by confocal microscopy. The excitation wavelength for Alexa was 488 nm, and the emission wavelength was 515 nm; for Syto 83 the excitation and emission wavelengths were 543 and 559 nm, respectively.

Immunoelectron microscopy. A suspension of 5×10^8 *L. kirschneri* was washed in 5 mM MgCl$_2$-PBS and incubated for 1 h in 33% preimmune, anti-LipL41, or anti-LipL32 antiserum (17). Bacteria were washed in 5 mM MgCl$_2$-PBS, fixed in 0.75% glutaraldehyde in 100 mM cacodylate acid buffer (pH 7.0), and washed in 0.15 M NaCl–10 mM CaCl$_2$–10 mM MgCl$_2$ and then applied to electron microscopy grids by the single-droplet technique as described previously (17). Grids were blocked with 1% bovine serum albumin in PBS, incubated for 10 min on goat anti-rabbit antibody–colloidal gold (10-nm particles; Sigma) and stained with 2% uranyl acetate. Organisms were selected at random by scanning the grid at low power and particles bound per organism were recorded. The results were analyzed for statistical significance by using the *t* test for two samples, assuming unequal variance.

Production of polyclonal antisera. The affinity-captured surfaceome obtained from 10^{11} *L. interrogans* serovar Lai cells was buffer exchanged on an Amicon YM10 membrane into 800 μl of sterile PBS (pH 7.2). An injectable suspension was made by mixing the affinity-captured surfaceome with an equal volume of Alhydrogel (Sigma). Four BALB/c mice that were negative for leptospiral antibodies were injected intraperitoneally with 200 μl of the suspension twice, 2 weeks apart. After a further 2 weeks, blood for serum was collected from the retro-orbital plexus. Antiserum to FlaA1 was prepared as follows: ExTaq (Takara) was used in the PCR to amplify the portion of the *flaA1* gene beginning with the first residue after the signal peptide. The 5′ primer contained the nucleotide sequence coding for the nine amino acids following the FlaA1 signal peptide, including an XhoI restriction endonuclease site (underlined): 5′-GA CTC GAG AAT GGA CAA AAC ATC ATC AAA GGC AAA C-3′. The 3′ primer contained the nucleotide sequence downstream from the *flaA1* stop codon, including a HindIII restriction endonuclease site (underlined): 5′-CCA AAG CTT AGA GAG AAG GAG AAA GCC TCA GAA AAG-3′. *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA was used as a template. The amplified *flaA1* gene was digested with the aforementioned enzymes and ligated into pRSETA (Invitrogen). The resulting construct, pRSETa-FlaA1, was transformed into *E. coli* BLR(DE3)/pLysS (Novagen) and expressed. The His$_6$-FlaA1 fusion protein was purified by affinity chromatography using Ni^{2+}-NTA-agarose (QIAGEN) and loaded onto an SDS-PAGE gel. After electrophoresis, the His$_6$-FlaA1 band containing ∼150 μg of protein was excised from the gel, desiccated, ground to powder, mixed with Freund complete adjuvant, and inoculated subcutaneously and intramuscularly into a New Zealand White male rabbit (Harlan). Additional immunizations with ∼150 μg of His$_6$-FlaA1 fusion protein in Freund incomplete adjuvant were given at 4 and 8 weeks after primary immunization. The rabbit was bled 10 weeks after the primary immunization.

Immunoblotting. Proteins were transferred onto Immobilon-P membranes with a Trans-Blot electrophoretic transfer cell. The membranes were incubated with a 1/20,000 dilution of rabbit anti-LipL21 (10) or 1/1,000 dilution of mouse anti-surfaceome. Binding of antibodies was detected by using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Silenus Laboratories). Immunoblots were developed by using the ECL Western blot detection system (Amersham) and visualized with Hyperfilm (Amersham).

RESULTS

Visualization of the leptospiral surfaceome. Viable leptospires were labeled with the water-soluble, membrane-impermeable, biotinylation reagent Sulfo-NHS-LC-Biotin, and the biotinylated products were visualized by one-dimensional gel electrophoresis (1-DGE) and 2-DGE (Fig. 1). Both techniques revealed selective labeling of a clearly defined subset of leptospiral proteins compared to leptospiral cells disrupted by sonication and labeled under identical conditions. This result was consistent with selective biotinylation of only those proteins exposed on the surface of the leptospiral cell. The cell surface profiles obtained were consistent and reproducible, with identical profiles observed when the procedure was conducted by different experimenters in laboratories in different countries. The images obtained after 2-DGE revealed chains of pI isoforms that are not observed after corresponding separation of unlabeled proteins, suggesting that these isoforms represent protein species that have incorporated various amounts of the biotin label.

Affinity capture and identification of surfaceome components. Several detergents were assessed for their ability to solubilize the leptospiral surfaceome compared to SDS; Triton X-100 was observed to effectively solubilize all of the surfaceome constituents (data not shown). To identify the selectively labeled surface proteins, biotinylated intact leptospires were solubilized with Triton X-100, and the products were purified by affinity chromatography with immobilized streptavidin. The affinity-captured products were analyzed by 1-DGE and 2-DGE. Spots were then excised from the gel, digested with trypsin, and subjected to MALDI-TOF MS (Fig. 2 and Table 1), which resulted in the identification of eight proteins. Of these, LipL21 and LipL41 were previously shown to be surface exposed (10, 38). A further two proteins, LipL32 and p31$_{LipL45}$, are known constituents of the leptospiral outer membrane. LipL32 gives rise to a number of pI and mass isoforms within the membrane (8) but, interestingly, we identified only the intact LipL32 and the LipL32.16 mass isoforms on the cell surface. Unexpectedly, we identified the flagellar subunit protein FlaB and the heat shock protein GroEL in the surfaceome preparation (see Discussion). Q8F8Q0 is a predicted outer membrane protein but has not been observed experimentally. Finally, no peptide mass matches were obtained for two proteins that we were therefore unable to identify (Fig. 2). Notably, LipL36, which is known to be anchored to the inner leaflet of the outer membrane (16, 38), was not detected by surface biotinylation. Likewise, surface biotinylation did not detect LipL31 or ImpL63, which are known to be components of the leptospiral inner membrane (17).

Assessment of LipL32 surface exposure by whole-cell ELISA. The standard ELISA plate coating buffer, 0.05 M sodium carbonate (pH 9.6), was not used to bind leptospires to microtiter wells because of concerns that the buffer may alter the integrity of the outer membrane (17). As an alternative, leptospiral cultures were added directly to microtiter wells, but even after prolonged incubation only low levels of adherence were observed. Consequently, microtiter wells were coated with the LPS-specific monoclonal antibody F71C2 to promote the capture of intact leptospires. Antisera to the abundant cytoplasmic protein GroEL and the inner membrane protein
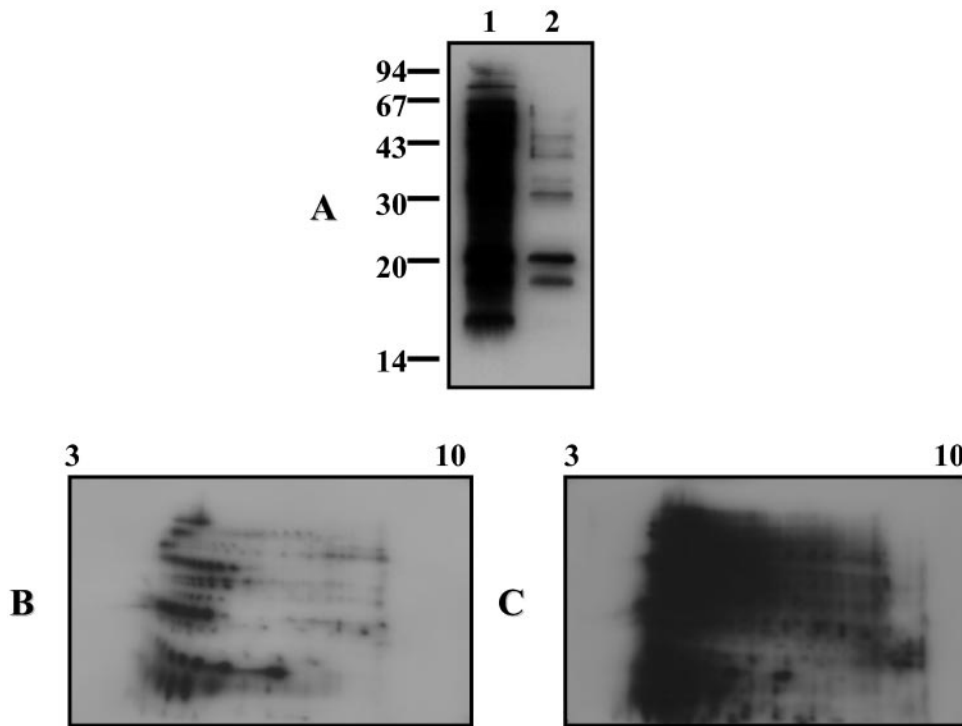
FIG. 1. (A) Biotin labeling of sonicated leptospires (lane 1) or intact leptospiral cells (lane 2). Biotinylated products were analyzed by SDS-PAGE and detected with streptavidin-peroxidase. The positions of standard molecular mass markers (in kilodaltons) are shown on the left. (B) 2-DGE (pH 3 to 10) of biotin-labeled intact leptospiral cells. (C) 2-DGE (pH 3 to 10) of a biotin-labeled leptospiral sonicate. The pI isoforms apparent on the 2-DGE separations are artifactual, arising from charge-induced changes relative to the number of biotin molecules attached to each protein.

LipL31 were subsequently applied to monitor the integrity of the leptospiral membranes. Antisera to both of these membrane markers showed very low background reactivity that did not increase with an increased number of leptospires per well (Fig. 3, top panel) or increased antibody concentration (Fig. 3, bottom panel). However, when wells were coated with leptospiral sonicates, both of these antisera resulted in high levels of reactivity that increased proportionally with increased cell and antibody concentrations (data not shown), indicating that the leptospiral membranes remained intact during the analysis.
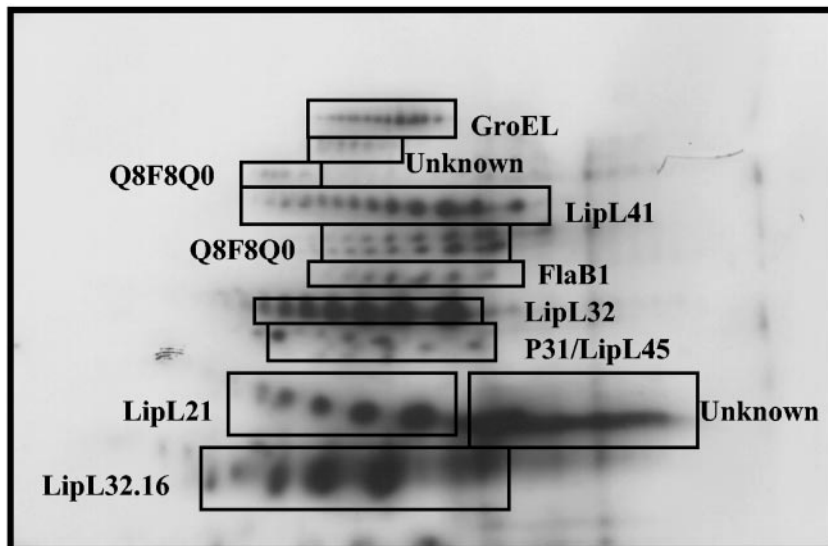

FIG. 2. Identification of leptospiral surfaceome proteins. Boxes indicate series of spots that were all identified as the same protein by MALDI-TOF MS (Table 1).

TABLE 1. Surface-labeled proteins identified by MALDI-TOF MS

Protein	No. of matching peptides	% Coverage
GroEL	13	29.5
Q8F8Q0	14	42.9
LipL41	6	20.8
FlaB1	15	49.8
LipL32	7	33.5
LipL45	7[a]	17.4
LipL32.16	5	23.3
LipL21	ND[b]	ND[b]

[a] All peptides were from the p31 fragment of LipL45.
[b] ND, not determined. LipL21 was identified by immunoblotting with specific antiserum.

When antisera to LipL32 and the surface-exposed protein LipL41 were applied to the wells, high levels of reactivity that increased proportionally with increased cell and antibody concentrations were observed (Fig. 3), confirming the surface exposure of the LipL32 protein.

Demonstration of LipL32 surface exposure by surface immunofluorescence. Viable intact leptospires were attached to slide chambers coated with an LPS-specific monoclonal antibody and subsequently exposed to antisera specific for either the cytoplasmic heat shock protein GroEL, the periplasmic flagellar sheath protein FlaA1, the surface-exposed LipL41, or the MOMP LipL32. Prior to the addition of these antisera, organisms were either left intact or permeabilized by immersion in methanol. The integrity of the leptospiral membranes was assessed by using antisera to the flagellar sheath protein FlaA1 (Fig. 4) and GroEL (data not shown), which did not label the bacteria unless the organisms were methanol permeabilized prior to antibody probing. Immunofluorescence of both permeabilized and intact organisms was observed when either the positive control anti-LipL41 sera (data not shown) or the anti-LipL32 (Fig. 4.) sera were used, confirming that LipL32 is exposed on the leptospiral cell surface.

Surface localization of LipL32 by immunoelectron microscopy. Whole-mount immunoelectron microscopy was performed with antisera to LipL32 and LipL41. Preimmune serum was used as the negative control, and the integrity of the outer membrane of the organisms was verified by visual inspection of the electron micrographs. Ten organisms incubated in preimmune serum, ten organisms incubated in LipL32 antiserum, and ten organisms incubated in LipL41 antiserum were quantified for surface colloidal gold binding. Organisms incubated in preimmune serum had a mean of 1.0 particles bound per organism compared to 10.8 particles bound per organism incubated in anti-LipL32 antiserum ($P < 0.000001$) and 5.1 particles bound per organism incubated in anti-LipL41 antiserum ($P < 0.000001$). Colloidal gold particles were randomly distributed on the leptospiral surface. The limited amount of colloidal gold particle binding was anticipated based on the results of previous immunoelectron microscopy studies of leptospiral outer membrane proteins (14, 26). Representative electron micrographs showing surface localization of LipL32 are shown in Fig. 5. Immunoelectron microscopic localization to the leptospiral surface provides ultrastructural evidence consistent with the hypothesis that LipL32 is a surface-exposed, outer membrane protein.

Lack of change in surfaceome under different conditions. Surface protein profiles were compared from leptospires grown under several conditions in vitro. The conditions examined included a range of cell densities from 10^7 to 10^9/ml, growth at 30 and 37°C, growth in 10% serum, and growth in 10% urine. No changes in composition or the relative abundance were observed (data not shown).

Immunization with affinity-captured surfaceome. Affinity-captured surfaceome proteins were mixed with Alhydrogel adjuvant and used to immunize BALB/c mice; the resultant antisera were examined by immunoblotting against a range of heterologous *Leptospira* species and serovars (Fig. 6). Sera recognized similar bands in all serovars reconstituting the protein profile of the surfaceome, with a slight difference in pattern seen in *L. fainei* serovar Hurstbridge. Interestingly, sera reacted with a protein of >94 kDa present in the homologous serovar Lai but absent in all other serovars, which was not detected by 2-DGE.

DISCUSSION

The surface of the bacterial cell forms the interface between pathogen and host and constitutes the site of interaction with host tissues during infection. For extracellular pathogens the bacterial cell surface is also the target for a protective host immune response. Recently, there has been interest in defining the subset of bacterial proteins present on the cell surface. For example, Montigiani et al. (30) used flow cytometry and 2-DGE–MS to define up to 53 proteins exposed on the surface of *Chlamydia pneumoniae*. With an approach similar to that in our study, Sabarth et al. (36) used surface biotinylation and MS to identify 18 of 82 putative surface-exposed proteins of *Helicobacter pylori*; 9 of these were previously shown to be surface exposed. However, in that study outer membranes were isolated subsequent to cell surface labeling and prior to electrophoretic separation, making it extremely difficult to assess the specificity of the labeling. Similar approaches have been used to profile the cell surface proteome of mammalian cells (20).

We have shown the surfaceome of *Leptospira* to consist predominantly of a relatively small number of proteins, most of which have been previously identified. It should be noted that determining the relative surface abundance via the method outlined is an approximation, due to the possibility of lysine residues (the target of the biotinylation reaction) being buried in the protein structure and not labeled efficiently. The three most extensively labeled proteins are lipoproteins and thus have no transmembrane regions in which lysine residues may be inaccessible by virtue of their membrane location. However, the relative abundance of proteins that take up beta-barrel confirmations in the outer membrane may be underestimated since labeling is dependent on the presence of lysines in the external loops. Taking into consideration these caveats, the three major proteins in order of relative abundance on the cell surface are LipL32, LipL21, and LipL41. It is significant that LipL32 and LipL41 have been reported as stimulating partial immunoprotection against leptospirosis in animal models (6, 18), a finding consistent with their surface location. LipL32 has been shown to undergo processing and modification, resulting in a range of pI and molecular mass isoforms (8). Interestingly, only two forms of LipL32 were detected on the leptospiral
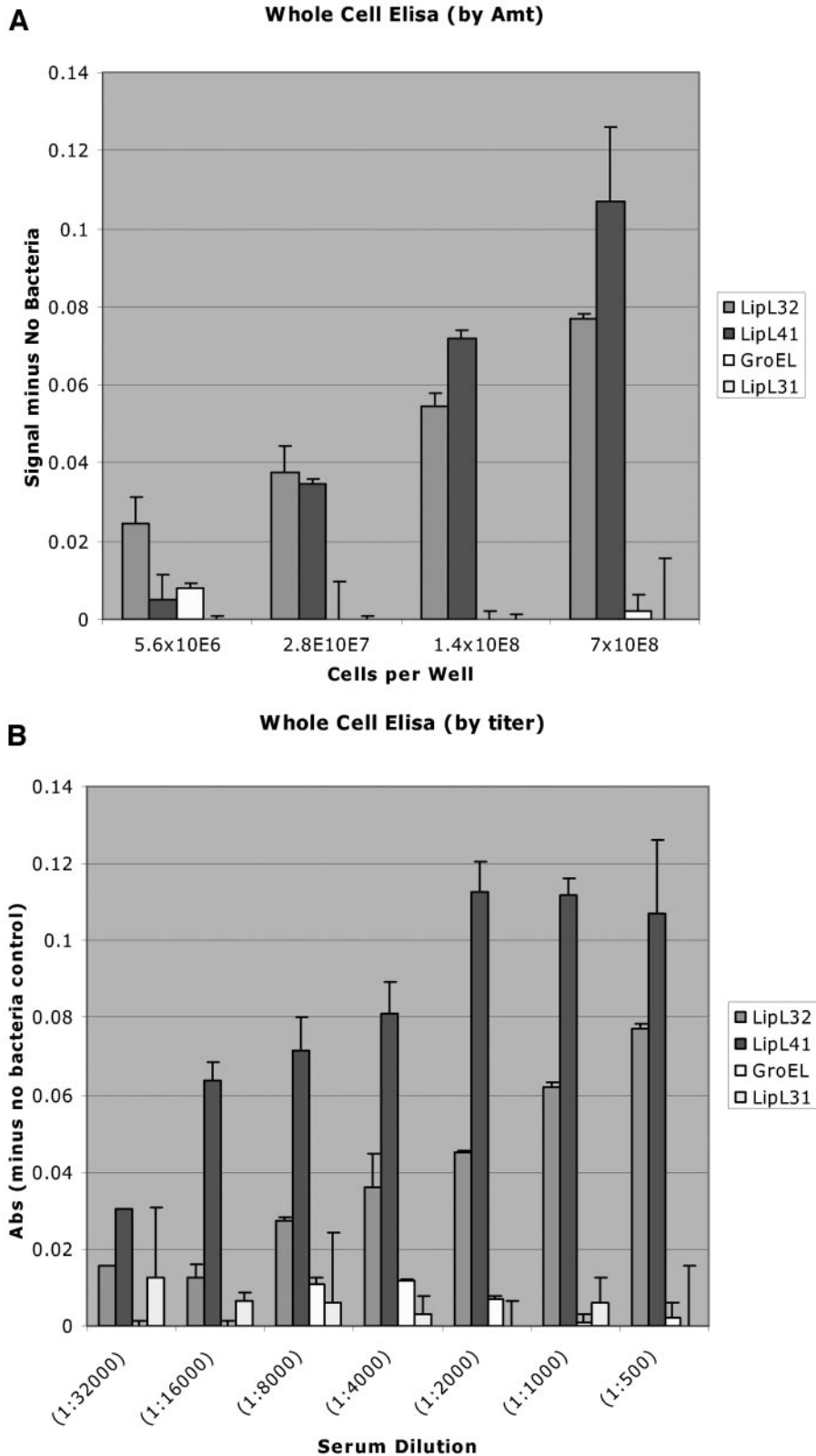
FIG. 3. Detection of *L. kirschneri* surface labeling by LipL32 and LipL41 antiserum and whole-cell ELISA. (Top) Whole-cell ELISA reactivity with a 1:500 dilution of antisera to the MOMP LipL32, the surface-exposed LipL41, the cytoplasmic heat shock protein GroEL, and the cytoplasmic membrane protein LipL31, with various concentrations of cells per well. (Bottom) Whole-cell ELISA reactivity with 7×10^8 cells per well with various dilutions of antisera to LipL32, LipL41, GroEL, and LipL31.
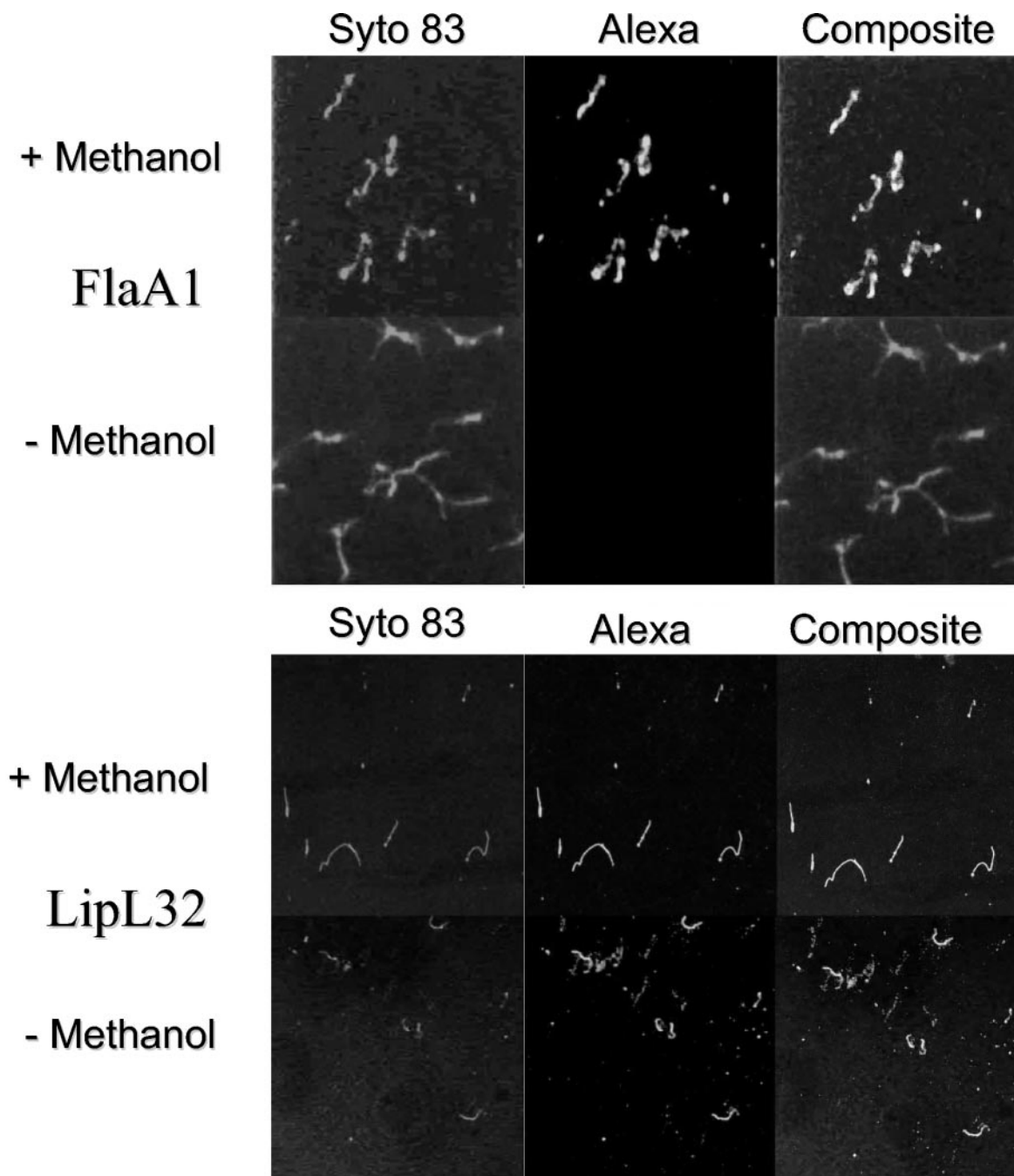
FIG. 4. LipL32 antiserum stains intact *L. kirschneri* by indirect immunofluorescence. *L. kirschneri* strain RM52 was fixed to microscope slides using an LPS-specific monoclonal antibody and probed with antisera to FlaA1 and LipL32 with or without methanol fixation, followed by incubation with Alexa-conjugated mouse anti-rabbit immunoglobulins. The locations of bacteria were identified by using the DNA counterstain Syto 83.

surface, viz. the full-length LipL32 and the 16-kDa molecular mass isoform LipL32.16. The biological significance of this is unknown. The protein Q8F8Q0 was annotated in the *L. interrogans* serovar Lai genome as a probable glycosyl hydrolase (35). However, our bioinformatics analysis indicated 42% similarity to an autotransporter from *Vibrio vulnificus* and a possible chitinase motif. The Q8F8Q0 sequence indicates a signal peptide, predicted amphipathic beta sheet, and an absence of

transmembrane alpha helices, features consistent with an integral, transmembrane, outer membrane protein (9). This protein may thus represent a new integral outer membrane protein which would be only the second such protein identified in *Leptospira*.

Due to ongoing controversies in the field of spirochete research regarding the surface exposure of outer membrane proteins (9) and the interest in LipL32 as a potential vaccinogen
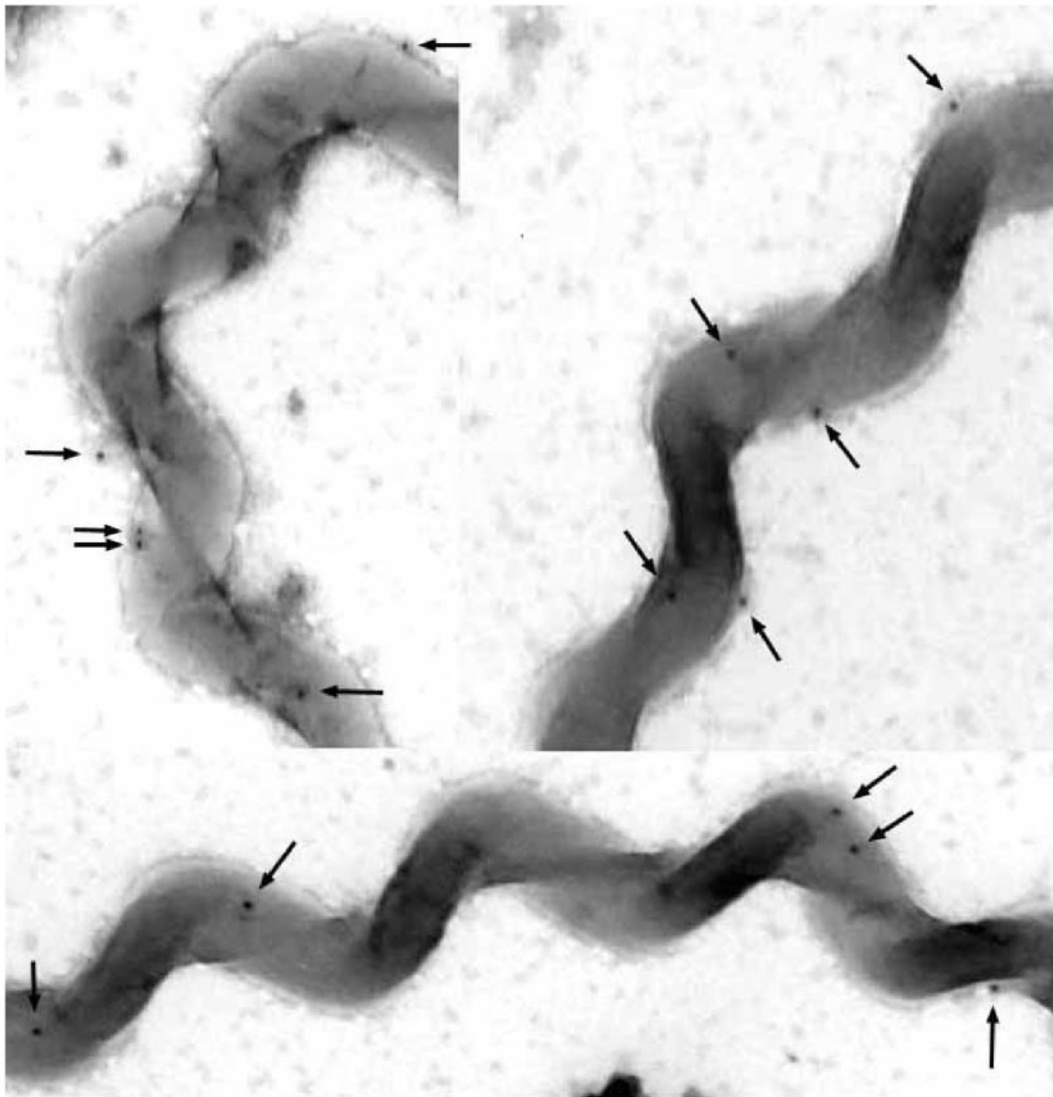
FIG. 5. Demonstration of LipL32 antiserum surface labeling of *L. kirschneri* by immunoelectron microscopy. Organisms incubated in LipL32 antiserum had a mean of 10.8 colloidal gold particles bound per organism compared to 1.0 colloidal gold particles bound per organism incubated in preimmune serum ($P < 0.0000001$). Representative organisms incubated in LipL32 antiserum are shown. Arrows indicate locations of colloidal gold particles. Bars, 100 nm.

(6), multiple methods were used to verify the surface exposure of LipL32. Two quantitative measures, whole-cell ELISA and immunoelectron microscopy, and one qualitative measure, surface immunofluorescence, were used. In contrast to surface biotinylation, all three of these assays are antibody based and make an assessment of antibody accessibility of the protein on the leptospiral cell surface. Surface-exposed major antigenic outer membrane proteins of bacterial pathogens typically have highly variable amino acid sequences, reflecting the pressure of the host immune system. LipL32 is one of the most highly expressed leptospiral proteins, is expressed during infection of the mammalian host and is highly antigenic (13, 15); >95% of patients with leptospirosis produce antibodies to LipL32 during infection (12). Despite this high level of expression and the antigenicity of the LipL32 molecule, sequence analysis of an entire spectrum of pathogenic *Leptospira* spp. revealed that the

LipL32 amino acid sequence was highly conserved, with a mean sequence identity of 99.1% (19). The whole-cell ELISA used to assess surface exposure via antibody accessibility provided some insight as to why the sequence of this highly expressed surface-exposed OMP remains invariable. When whole cells were substituted with an equivalent number of sonicated organisms in the whole-cell ELISA, the sonicates were found to be approximately 10 times more reactive (data not shown), suggesting that only a portion of the cellular LipL32 is accessible to antibody. Taken together with the finding that LipL32 was the most highly labeled protein using surface biotinylation, this seems to suggest that LipL32 is highly surface exposed but poorly accessible to larger molecules such as antibodies. This may result from steric hindrance of LipL32 epitopes by the carbohydrate side chains of the LPS that act like a "rainforest canopy" to dominate the leptospiral
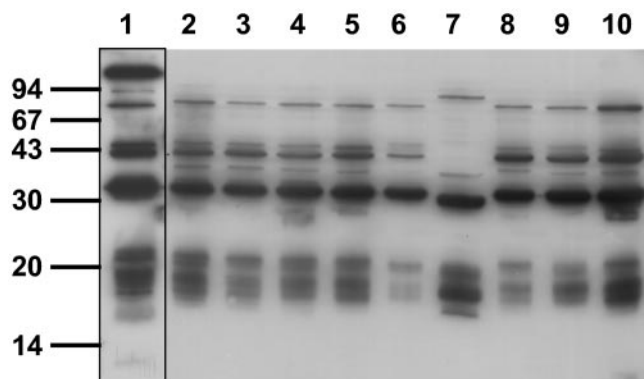
FIG. 6. Reaction of sera from mice immunized with affinity captured surfaceome proteins of serovar Lai with heterologous leptospiral serovars. Lanes: 1, *L. interrogans* serovar Lai; 2, *L. interrogans* serovar Australis; 3, *L. interrogans* serovar Canicola; 4, *L. interrogans* serovar Copenhageni; 5, *L. kirschneri* serovar Grippotyphosa; 6, *L. borgpetersenii* serovar Hardjobovis; 7, *L. fainei* serovar Hurstbridge; 8, *L. interrogans* serovar Pomona; 9, *L. borgpetersenii* serovar Tarassovi; 10, *L. interrogans* serovar Zanoni.

surface and obscure the protein antigens anchored in the membrane below. In support of this hypothesis, we have found that all of the leptospiral outer membrane proteins characterized thus far remain resistant to digestion with proteinase K or pronase when whole cells are exposed to concentrations of up to 500 μg/ml (unpublished data). An alternate but less parsimonious explanation is that the polyclonal antiserum raised against recombinant LipL32 contains limited antibody species that react with the native LipL32 in its membrane context.

Two other labeled proteins of ca. 20 and 55 kDa were observed but could not be identified by MS. Possible reasons for the inability to identify these proteins include resistance to trypsin digestion (7, 40), the mass-altering effect of biotinylation, or the presence of unknown posttranslational modifications, resulting in a failure to derive peptide mass matches. However, the 20-kDa protein demonstrated a significant degree of surface labeling and may therefore represent an additional as-yet-uncharacterized, surface-exposed protein. The outer membrane lipoprotein LipL48 that has been described previously (8, 28) was not identified as a component of the surfaceome. This suggests that LipL48 is located in the inner leaflet of the outer membrane, as previously demonstrated for LipL36 (16).

A low level of labeling of $p31_{LipL45}$, GroEL, and FlaB1 was also observed. The labeling of these proteins needs to be put into the context of the extraordinary sensitivity of this system. The p31 fragment of LipL45 has been demonstrated to be membrane associated (28) and is therefore a plausible minor constituent of the surfaceome that awaits validation. GroEL is a chaperone that engages in numerous protein-protein interactions; a low level of labeling of this protein at the cell surface is probably legitimate but is likely to result from release of this protein from lysed cells prior to binding to the surface of intact cells. The low level labeling of the major flagellar subunit protein FlaB1 is puzzling and cannot yet be explained. The absence of labeling of the flagellar sheath proteins FlaA1 and FlaA2, the cytoplasmic membrane markers ImpL63 and LipL31 (17), and the remainder of the proteome precluded the

possibility that the biotinylation reagent overcame the permeability barrier of the outer membrane. Previously, we were unable to identify the outer membrane porin OmpL1 due to its basic pI and relatively low abundance (8); the same was true in the present study. One of the constraints of gel-based proteomics is that it is difficult, without using separate specialized protocols, to identify very basic protein and large proteins (5). The high level of expression of some proteins creates a dynamic range problem that limits the identification of proteins that are expressed at low levels. Hence, although our approach has been the most global to date we believe there are more leptospiral surface proteins that remain to be identified.

It has been shown that the expression of some leptospiral outer membrane proteins may vary under different conditions. For example, LipL36 is not expressed in vivo (16) and is downregulated at 37°C and at low iron concentrations (8). In this context, it was perhaps unexpected that the expression of the surfaceome constituents remained unchanged under all of the conditions we investigated. The expression of the p31/LipL45 fragment has previously been demonstrated to be growth phase regulated; despite this, the total amount of protein on the cell surface appears to remain constant. The prospect that the expression of the surfaceome may vary under other conditions of course remains a possibility. There is evidence accumulating that the surfaceome compositionally differs in leptospires recently isolated from mammalian hosts. A pair of large leptospiral bacterial-immunoglobulin-like outer membrane proteins termed LigA and LigB, whose expression declines after laboratory passage, was recently identified (26, 32). In addition, the expression of the Ligs has recently been found to be dependent on osmolarity (27). Future studies examining expression of the surfaceome will utilize organisms recently isolated from the host and will also investigate the effects of osmolarity.

Surfaceome components stimulated an antibody response in mice that recognized similar proteins present in a range of pathogenic serovars, thus reconstituting the surfaceome protein profile. A notable exception was *L. fainei* serovar Hurstbridge, for which no band of 41 kDa was recognized, possibly suggesting that this strain may either not possess the surface lipoprotein LipL41 or not express it on its cell surface. *L. fainei* has been termed an "intermediate pathogen" (10a, 34), and the loss of LipL41 expression on the cell surface may correlate with the reduced pathogenicity of this organism. Interestingly, the anti-surfaceome sera reacted with a protein of >94 kDa present in the homologous serovar Lai but which was not recognized in any of the other serovars examined. The protein was clearly present in low abundance and was not detected by 2-DGE and MS. Its absence in 2-DGE analysis was not due to its pI, molecular mass, or other properties that may occasionally affect detection, since no protein of this size was detected in standard SDS-PAGE analysis of affinity-captured surfaceome components (data not shown). This protein may therefore represent a serovar Lai-specific surface component or may be a low-abundance nonsurface protein. In the absence of protein identification data we cannot favor either explanation. Clearly, this protein is highly immunogenic because it was below the level of detection after gel electrophoresis or after blotting of biotinylated protein samples, yet it was still able to elicit the production of antibodies.

The surfaceomics approach is particularly amenable to protein expression profiling using small amounts of sample, with only ca. 10^7 cells required for a 2-D gel, thereby overcoming one of the limitations of this approach, i.e., the lack of sufficient material for analysis. This becomes a particular problem in attempts to study bacteria derived from host tissues, a problem that should be alleviated by the surface-labeling approach and which should therefore permit analysis of the surfaceome of leptospires obtained from infected tissues. In addition, immunization of mice with the surfaceome stimulated heterologous antibodies to leptospiral surface components, thus providing a reagent for further investigations into the role of surface proteins in immunity to leptospirosis.

ACKNOWLEDGMENTS

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