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Molecular Cloning and Sequence Analysis of the Gene Encoding OmpL1, a Transmembrane Outer Membrane Protein of Pathogenic *Leptospira* spp.

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Pathogenic *Leptospira* spp. are spirochetes that have a low transmembrane outer membrane protein content relative to that of enteric gram-negative bacteria. In a previous study we identified a 31-kDa surface protein that was present in strains of *Leptospira alstoni* in amounts which correlated with the outer membrane particle density observed by freeze fracture electron microscopy (D. A. Haake, E. M. Walker, D. R. Blanco, C. A. Bolin, J. N. Miller, and M. A. Lovett, Infect. Immun. 59:1131–1140, 1991). The N-terminal amino acid sequence was used to design a pair of oligonucleotides which were utilized to screen a lambda ZAP II library containing *Eco*RI fragments of *L. alstoni* DNA. A 2.5-kb DNA fragment which contained the entire structural *ompL1* gene was identified. The structural gene deduced from the sequence of this DNA fragment would encode a 320-amino-acid polypeptide with a 24-amino-acid leader peptide and a leader peptidase I cleavage site. Processing of OmpL1 results in a mature protein with a predicted molecular mass of 31,113 Da. Secondary-structure prediction identified repeated stretches of amphipathic beta-sheets typical of outer membrane protein membrane-spanning sequences. A topological model of OmpL1 containing 10 transmembrane segments is suggested. A recombinant OmpL1 fusion protein was expressed in *Escherichia coli* in order to immunize rabbits with the purified protein. Upon Triton X-114 extraction of *L. alstoni* and phase separation, anti-OmpL1 antiserum recognized a single band on immunoblots of the hydrophobic detergent fraction which was not present in the hydrophilic aqueous fraction. Immunoelectron microscopy with anti-OmpL1 antiserum demonstrates binding to the surface of intact *L. alstoni*. DNA hybridization studies indicate that the *ompL1* gene is present in a single copy in all pathogenic *Leptospira* species that have been tested and is absent in nonpathogenic *Leptospira* species. OmpL1 may be the first spirochetal transmembrane outer membrane protein for which the structural gene has been cloned and sequenced.

Leptospirosis is a widespread zoonosis caused by members of the genus *Leptospira*. These highly invasive spirochetal pathogens are capable of infecting a broad range of mammalian hosts through either direct contact with an infected animal or indirect contact with soil or water contaminated with urine from a chronically infected animal. In humans, acute leptospirosis accounts for roughly 10% of hospitalizations for acute febrile illness in tropical areas of the world (11). Leptospirosis is an important cause of morbidity in U.S. military personnel, occurring in 2 to 8% of soldiers undergoing jungle training in Panama (36). Leptospirosis also causes significant abortion, stillbirth, infertility, decreased milk production, and death in livestock (37). Control efforts have been hampered by the fact that virulent leptospires are persistently shed from the urinary tracts of wildlife and livestock and subsequently are able to survive in the environment. Currently available vaccines for prevention of leptospirosis produce only short-term immunity and do not provide cross-protection against many of the 170 different serovars of pathogenic *Leptospira* spp. Because of a growing appreciation of leptospiral diversity, there are now six pathogenic species and three nonpathogenic species within the genus *Leptospira* (47).

Freeze fracture electron microscopy has shown that pathogenic *Leptospira* spp. belong to a group of virulent spirochetes, including *Treponema pallidum* and *Borrelia hermsii*, that have a low density of outer membrane proteins (OMPs) relative to that in enteric gram-negative bacteria (12, 18, 29, 43, 44). For treponemes and leptospires there appears to be a correlation between low OMP density and virulence (12, 44). The kinetics of complement activation and outer membrane particle aggregation indicate that *T. pallidum* OMPs are important targets of treponemicidal antibody (4). Because of outer membrane fragility and the fact that OMPs are present in small amounts, there have been no reports identifying spirochetal proteins that span the outer membrane. OMPs of gram-negative bacteria are of great interest because they are located at the cell surface, where bacterial pathogens interact with the host (28). OMPs may play a role in bacterial pathogenesis by acting as (i) adhesins (2, 16, 26, 31), (ii) targets of bactericidal antibody (10, 27, 32), (iii) porins (10, 17, 23, 25), and (iv) receptors for soluble molecules, such as siderophores (34) and complement proteins (14). There is also evidence that OMPs can elicit protective antibodies against disease (13, 32).

In order to identify potential leptospiral OMP candidates, we performed a comprehensive study of the surface components of a grippotyphosa serovar, isolated during an outbreak of swine leptospirosis, which has now been assigned to the species *Leptospira alstoni* (12). One approach involved immunoprecipitation experiments which identified

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31-, 41-, and 44-kDa protein antigens on the surface of intact *L. alstoni*. In that study, the molecular mass of the 31-kDa protein antigen was incorrectly predicted to be 33 kDa (12). The 41- and 44-kDa proteins were present in equal amounts in virulent and culture-attenuated *L. alstoni*, while expression of the 31-kDa protein was positively correlated with OMP density as determined by freeze fracture electron microscopy. In this study, we have cloned and sequenced the structural gene for the 31-kDa protein. The 31-kDa protein has been designated OmpL1 from its hydrophobic behavior in Triton X-114, preliminary localization studies using specific anti-OmpL1 antiserum, and secondary-structure prediction of the deduced amino acid sequence. A single copy of the *ompL1* gene is present in all six pathogenic *Leptospira* species tested and is absent from all nonpathogenic species.

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MATERIALS AND METHODS

Leptospiral strains. Virulent and culture-attenuated *L. alstoni* serovar grippotyphosa (strain RM52) were received from C. A. Bolin (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa). This strain was originally isolated from material submitted to the Veterinary Diagnostic Laboratory at Iowa State University during an outbreak of swine abortion in 1983 (38). Samples of the isolate were either stored in liquid nitrogen (1) or passaged weekly or biweekly in liquid EMJH medium (20). The virulent strain had been passaged fewer than five times. As described previously (12), the attenuated strain has been passaged more than 200 times since 1983. The minimum lethal inoculum was $<10^4$ for the virulent strain and $>10^9$ for the attenuated strain as determined by intraperitoneal inoculation into weanling hamsters. The minimum infectious inoculum was $<10^4$ for the virulent strain and 10^7 for the attenuated strain.

Escherichia coli. *E. coli* DH5α [*supE44 ΔlacU169* (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 λZap 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).

SDS-PAGE and immunoblotting. Samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were boiled for 10 min in final sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, and 2% SDS and then were separated by electrophoresis on 2.5% stacking and 10 to 15% linear gradient polyacrylamide gels with the discontinuous buffer system of Laemmli (22). After electrophoresis, gels were transferred to nitrocellulose for immunoblotting (40). For antigenic detection on immunoblots, the nitrocellulose was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS)–0.1% Tween 20, incubated for 1 h with antiserum diluted 1:1,000 in PBS–0.1% Tween 20, and probed with donkey antirabbit antiserum conjugated to horseradish peroxidase (Amersham). Antigen-antibody binding was detected by using the Enhanced Chemiluminescence System (Amersham). Blots were incubated in Enhanced Chemiluminescence System reagents for 1 min and then exposed to XAR-5 film (Fuji).

OmpL1 isolation and N-terminal amino acid sequencing. The 31-kDa OmpL1 protein was prepared for N-terminal amino acid sequencing by surface immunoprecipitation as previously described (12). Culture-attenuated *L. alstoni* was used for immunization and purification because this strain contains more of the 31-kDa protein than the virulent strain. In brief, a New Zealand White male rabbit was immunized intramuscularly and subcutaneously with 5×10^8 culture-attenuated *L. alstoni* organisms in Freund's complete adjuvant. The rabbit was boosted at 6 and 12 weeks with 10^9 culture-attenuated *L. alstoni* organisms in Freund's incomplete adjuvant. Ten milliliters of antiserum obtained after the second boost was added to a leptospiral culture in the log phase of growth containing 5.6×10^{11} culture-attenuated *L. alstoni* organisms which were >99% motile. The suspension was then gently shaken for 2 h at 30°C. Agglutinated leptospires were pelleted at $2,000 \times g$ for 15 min, resuspended in 5 mM $MgCl_2$ in PBS, pelleted again at $2,000 \times g$ for 15 min, and then resuspended in 20 ml of 10 mM Tris HCl (pH 8.0)–10 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride–1 mM iodoacetamide. To this suspension was added 2 ml of protein-grade 10% Triton X-100 (Calbiochem), and then the suspension was gently agitated for 1 h at 4°C. The insoluble material was removed by centrifugation at $16,000 \times g$ for 10 min. To the supernatant was added 2 ml of 2% sodium deoxycholate, 100 μl of 20% SDS, and 500 μl of a slurry of staphylococcal protein A–Sepharose CL-4B (Sigma). This mixture was gently agitated for 1 h at 4°C. The staphylococcal protein A–Sepharose CL-4B–antibody–antigen complexes were pelleted at $500 \times g$ for 5 min, resuspended in 0.01% Triton X-100 in 10 mM Tris HCl (pH 8.0), and pelleted again at $500 \times g$ for 2 min. The pellet was then resuspended in final sample buffer, boiled for 15 min, and subjected to SDS-PAGE. Electrophoresed proteins were transferred to Trans-Blot PVDF Protein Sequencing Membrane (Bio-Rad, Richmond, Calif.) and submitted to the UCLA Protein Microsequencing Facility. N-terminal amino acid sequence analysis was performed on a Porton 1090-E gas phase sequenator with on-line detection of phenylthiohydantoin amino acids.

Southern blot analysis. *L. alstoni* genomic DNA was prepared by the method of Yelton and Charon (48). 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 (30). Filters were baked for 2 h at 80°C under vacuum and 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.

Two degenerate oligonucleotide probes, each 20 bp in length, were synthesized on the basis of the N-terminal amino acid sequence of the 31-kDa protein (Fig. 1). Oligonucleotide 1 was synthesized in the laboratory of Stephen Smale (Department of Microbiology and Immunology, UCLA School of Medicine). Oligonucleotide 2 was synthesized by Genosys Biotechnologies, Inc. (The Woodlands, Tex.). For degenerate oligonucleotide probes, the filters were washed at 47°C in 3.0 M tetramethylammonium chloride (Aldrich)–50 mM Tris (pH 8.0)–2.0 mM EDTA–1.0%

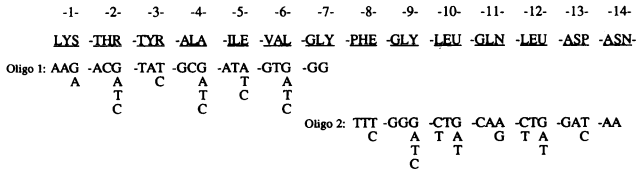

FIG. 1. N-terminal amino acid sequence of the native OmpL1 protein of *L. alstoni* serovar grippotyphosa, strain RM52, and degenerate oligonucleotides 1 and 2 used to probe Southern blots and screen the lambda ZAP II library (see text for details).

SDS as previously described (46). Southern hybridization was also performed with an *ompL1* gene fragment probe labeled with [^{32}P]dCTP by the random priming method (USB). For the *ompL1* gene fragment probe, the filters were washed first at medium stringency in 2× SSC at 55°C and then at high stringency in 0.1× SSC at 55°C.

Cloning and sequencing of the *ompL1* gene. *Eco*RI fragments of *L. alstoni* genomic DNA in the molecular weight range identified by Southern hybridization with the two oligonucleotide probes 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). Roughly 6,000 plaques were plated, transferred to filters in duplicate, and processed as previously described (30). The same oligonucleotide probe hybridization and washing conditions were used as described above for Southern hybridization. Recombinant pBluescript SK(−) clones were recovered from phage producing positive plaques by in vivo excision according to the manufacturer's instructions. After restriction mapping, appropriate DNA fragments were subcloned into pBluescript KS and sequenced at the UCLA Core DNA Sequencing Facility by the dideoxy chain termination method with fluorescein-labeled dideoxy nucleotides (Applied Biosystems).

DNA sequence analysis. DNA sequence information was analyzed by the DNA Strider program (24). Homology searches were performed with the FASTA and Profilesearch programs, which are found in the University of Wisconsin Genetics Computer Group, Inc., package, version 7.0 (8). Secondary-structure predictions were based upon analysis using the programs PEPPLOT and PLOTSTRUCTURE, which are also found in the Genetics Computer Group package. Hydrophobicity plots and beta-moment plots were generated by using the Moment program at the laboratory of David Eisenberg, Molecular Biology Institute, UCLA (9).

Immunization with the His_6-OmpL1 fusion protein. The pBluescript plasmid isolate containing the *ompL1* gene was digested with *Nde*I, filled in with Klenow fragment to generate blunt ends, digested with *Eco*RI, and ligated into pRSET (Invitrogen) digested with *Sma*I and *Eco*RI. The resulting construct, pRSET-*ompL1*, was transformed into *E. coli* JM109 (Invitrogen). Expression of the His_6-OmpL1 fusion protein was achieved by β-D-galactosidase induction (IPTG; Sigma) followed by infection with M13-T7 phage containing the T7 polymerase gene driven by the *E. coli lac* promoter. The His_6-OmpL1 fusion protein was separated from other insoluble materials by SDS-PAGE. The His_6-OmpL1 band containing 50 μg of protein was cut out of the acrylamide gel, dessicated, ground to powder, mixed with Freund's complete adjuvant, and inoculated subcutaneously and intramuscularly into a New Zealand White male rabbit. Additional His_6-OmpL1 fusion protein was solubilized in 6 M guanidine, purified by affinity chromatography using the Probond resin column (Invitrogen), and dialyzed in 10 mM Tris (pH 8). The secondary immunization was given 6 weeks after the primary immunization, using roughly 50 μg purified His_6-OmpL1 fusion protein in Freund's incomplete adjuvant. The rabbit was bled 2 weeks after the secondary immunization.

Triton X-114 extraction of *L. alstoni*. Triton X-114 extraction of *L. alstoni* was carried out as described previously (12). In brief, culture-attenuated *L. alstoni* cells were washed twice in PBS–5 mM $MgCl_2$ and extracted in 10 mM Tris (pH 8)–1 mM EDTA–1% Triton X-114 at 4°C. The insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. The Triton X-114 concentration in 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 and separated by SDS-PAGE (10%).

Immunoelectron microscopy of whole *L. alstoni* with anti-OmpL1 antiserum. A suspension of 2×10^7 culture-attenuated *L. alstoni* organisms in culture medium was incubated for 1 h in 33% heat-inactivated preimmune serum or anti-OmpL1 antiserum. The bacteria were then fixed in 0.25% glutaraldehyde, washed in 0.15 M NaCl–5 mM $CaCl_2$–5 mM $MgCl_2$, and applied to electron microscopy grids by the single-droplet technique as described previously (7). Grids were blocked with 0.5% bovine serum albumin–PBS, incubated for 10 min on staphylococcal protein A-colloidal gold (10-nm particles), and stained with 1% uranyl acetate. Organisms were selected at random by scanning the grid at low power, and the number of particles bound per organism was recorded. Results were analyzed for statistical significance by the *t* test for two samples, assuming unequal variance.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession number L13284.

RESULTS

Design of oligonucleotide probes and cloning of the *ompL1* gene. N-terminal amino acid sequence analysis of the mature 31-kDa protein made it possible to design two nonoverlapping degenerate oligonucleotide probes (Fig. 1). Codon bias was used for the leucine residues in the design of oligonucleotide 2 (1,152-fold degeneracy) but not for oligonucleotide 1 (768-fold degeneracy). The codon bias strategy reflected the low GC content of *Leptospira* spp. (19). The oligonucleotide probes independently identified a 2.5-kb *Eco*RI fragment by Southern hybridization of the *L. alstoni* genome. DNA fragments with a size range of 2.1 to 2.8 kb were cut out of an agarose gel, purified, and ligated into the lambda ZAP II vector (Stratagene). The oligonucleotide probes were used to independently screen a library of 4,800 plaques. Both oligonucleotide probes hybridized to the same 18 plaques. Six positive plaques were picked, replated, and reprobed in order to purify phage bearing the hybridizing DNA fragment. Purified phage were amplified and converted to pBluescript SK(−) plasmid form by in vivo excision and recircularization. All six pBluescript plasmids obtained in this way contained the same 2.5-kb *Eco*RI insert. A restriction map of the insert was constructed, and Southern hybridization using the oligonucleotide probes localized the area of hybridization to a 122-bp fragment near the *Nde*I site (Fig. 2).

Sequence analysis. An intact open reading frame was

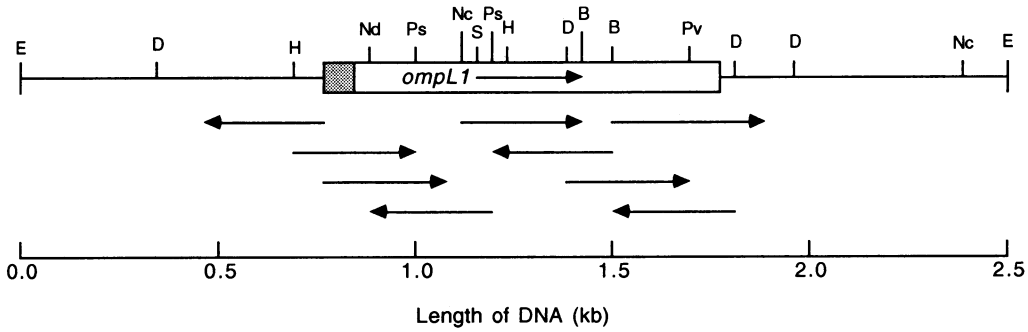

FIG. 2. Partial restriction map of the 2.5-kb *Eco*RI fragment containing the *ompL1* gene and strategy for determining the nucleotide sequence. The shaded area indicates the location of the OmpL1 leader peptide. The arrows below the map indicate the direction and extent of sequence analysis. Single letters above the map indicate the following restriction enzymes: *Eco*RI (E), *Hpa*II (H), *Nde*I (Nd), *Pst*I (Ps), *Nco*I (Nc), *Ssp*I (S), *Dra*I (D), *Bam*HI (B), and *Pvu*I (Pv).

identified 770 bp downstream from the *Eco*RI site. The *ompL1* structural gene consists of 960 bases encoding a protein of 320 amino acids. *E. coli*-like −35 (TTGCCG) and −10 (TCCAAT) promoter regions, and a consensus ribosome-binding site (AGGAG) are present upstream from the initiation codon (Fig. 3). As expected for proteins exported through the cytoplasmic membrane, the derived amino acid sequence begins with a 24-residue leader peptide, represented by the shaded area on the restriction map (Fig. 2) and the N-terminal peak on the hydrophobicity plot (Fig. 4A). The OmpL1 sequence conforms to the rules established for procaryotic leader peptides (42). The OmpL1 leader peptide has a basic amino-terminal region (including an arginine at position 3), a hydrophobic core (amino acids 5 through 18), and a carboxy-terminal region with a leader peptidase I cleavage site. It is also notable that a Leu-X-Y-Cys leader peptidase II cleavage site is not present, which argues that OmpL1 is not a lipoprotein. Immediately following the leader peptidase I cleavage site is a sequence of 13 amino acids that is identical to the sequence obtained by N-terminal amino acid sequence analysis of the native protein (Fig. 1). The 320-amino-acid protein has a predicted mass of 33,508 Da, which is similar to that observed experimentally. Downstream from the termination codon is an inverted repeat followed by four T residues, a configuration observed for rho-independent transcription terminators (Fig. 3). A search of the GenBank data base did not reveal amino acid sequence homologies. Following the leader peptide, there are no long alpha-helical hydrophobic membrane-spanning domains as found in cytoplasmic membrane proteins. The OmpL1 sequence does have characteristics in common with OMPs. Secondary-structure predictions identify repeated stretches of amphipathic beta-sheets consistent with OMP transmembrane segments, most of which are reflected as peaks on the beta-moment plot (Fig. 4B). A topological model of OmpL1 with 10 transmembrane segments is proposed (Fig. 5). Each of the transmembrane segments is 10 amino acids in length and conforms to an alternating pattern of hydrophobic residues. The terminal transmembrane segment has a histidine three amino acids from the carboxy terminus, a pattern seen in porins of *Neisseria* and *Bordetella* spp. (17, 23, 25) (Table 1). Five surface-exposed loops of various lengths, which contain all five regions with high surface probability, hydrophilicity, flexibility, and antigenic indices determined by using the PLOTSTRUCTURE program (data not shown), are proposed. Four periplasmic loops, which are typical of those found in OMPs in that they are short and contain turn promoting residues, are proposed.

Occurance of the *ompL1* gene in *Leptospira* spp. To address the frequency and distribution of the *ompL1* gene, we performed Southern hybridization analysis with an 834-bp *Nde*I-*Pvu*I-internal fragment of the *ompL1* gene encoding 94% of the mature protein. Figure 6 shows the results of probing genomic digests from representative strains from most of the known pathogenic and nonpathogenic *Leptospira* spp. A single copy of the *ompL1* gene is present in all six pathogenic *Leptospira* species tested (Fig. 6A). When the same blot was washed under high-stringency conditions, the greatest degree of similarity to the *ompL1* gene of strain RM52 was found within the species *L. alstoni* (Fig. 6B). The gene was not detected in *Leptospira biflexa*, *Leptospira meyeri*, or *Leptospira wolbachii*, the three known nonpathogenic *Leptospira* species, or in the related nonpathogen *Leptonema illini*. The *ompL1* gene may be a marker for pathogenicity. However, an alternative explanation for these results is that they reflect the fact that there is less than 10% DNA-DNA hybridization between the pathogenic and nonpathogenic *Leptospira* spp.

Generation of specific anti-OmpL1 antiserum. The 1,600-bp *Nde*I-*Eco*RI fragment ligated into pRSET encodes essentially the entire mature OmpL1 protein. The fusion protein expressed by pRSET-*ompL1* contains a 41-amino-acid His_6-binding site at the amino terminus of OmpL1. The six histidines allowed for pH-dependent affinity purification of the fusion protein on a Probond (Invitrogen) nickel resin column to the exclusion of *E. coli* proteins. The pRSET fusion protein is under T7 promoter control. After transformation of pRSET-*ompL1* into *E. coli* JM109 and infection with M13-T7 phage, milligram quantities of the His_6-OmpL1 fusion protein were produced in the presence of IPTG (data not shown). The majority of the *E. coli* host proteins were released after several freeze-thaw cycles in lysis buffer, while >90% of the insoluble pellet consisted of the His_6-OmpL1 fusion protein. Antiserum from the rabbit immunized and boosted with purified His_6-OmpL1 fusion protein reacted only with the 31-kDa antigen on immunoblots of *L. alstoni* separated by SDS-PAGE (Fig. 7). Preimmune serum was nonreactive (data not shown).

Localization of OmpL1 in *L. alstoni* by Triton X-114 fractionation and by immunoelectron microscopy. We analyzed the behavior of OmpL1 in the nonionic detergent Triton X-114 and tested the specificity of the anti-OmpL1 anti-

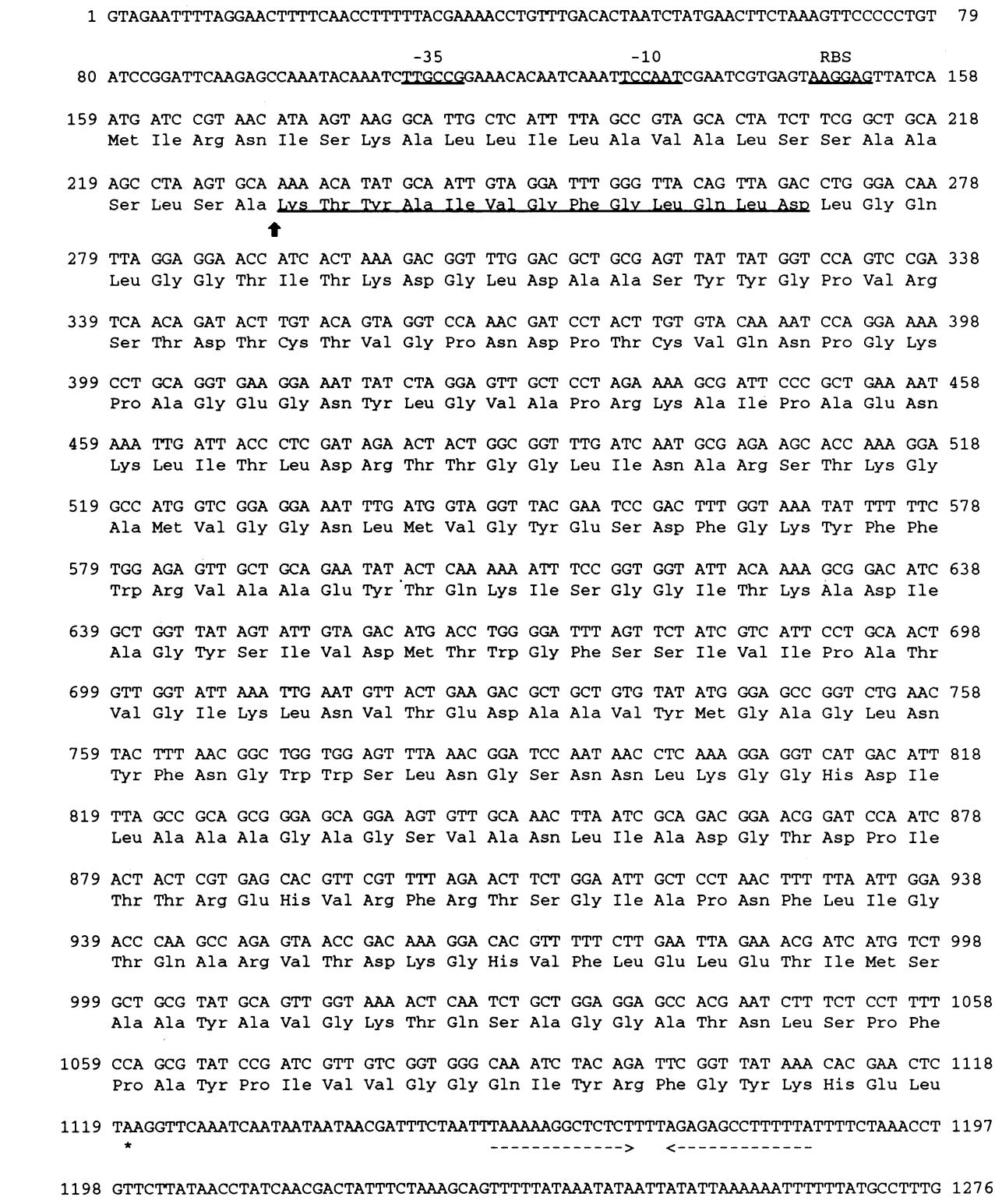

FIG. 3. Nucleotide sequence and derived amino acid sequence of *ompL1*. Putative −35 and −10 promoter regions are shown. The ribosome-binding site (RBS) is also shown. The leader peptidase I cleavage site is indicated by an arrow (⬆) and is followed by the N-terminal amino acid sequence determined from the native protein (underlined). The location of the TAA stop codon is indicated by an asterisk. An inverted repeat is indicated by the horizontal broken arrows from positions 1155 to 1185; this repeat may function as a rho-independent transcription terminator.

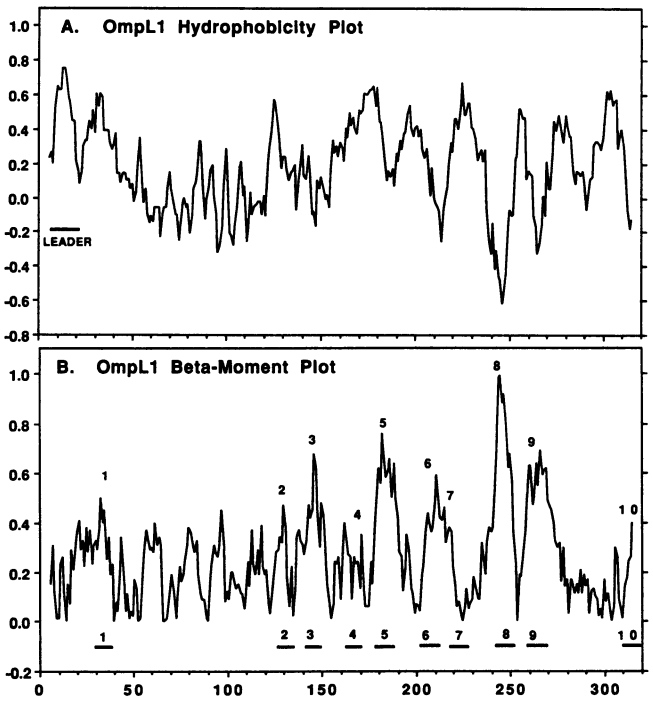

FIG. 4. Hydrophobicity and beta-moment plots of the derived amino acid sequence of OmpL1. The hydrophobicity plot reveals an N-terminal peak of 0.75 corresponding to the leader peptide. Following the leader peptide, there are no long alpha-helical hydrophobic membrane-spanning domains as found in cytoplasmic membrane proteins. Peaks on the beta-moment plot are regions where hydrophobic residues alternate with hydrophilic residues, indicating where transmembrane segments may occur in an OMP (see text). The locations of the 10 transmembrane segments on the suggested topological model of OmpL1 (Fig. 5) are shown.

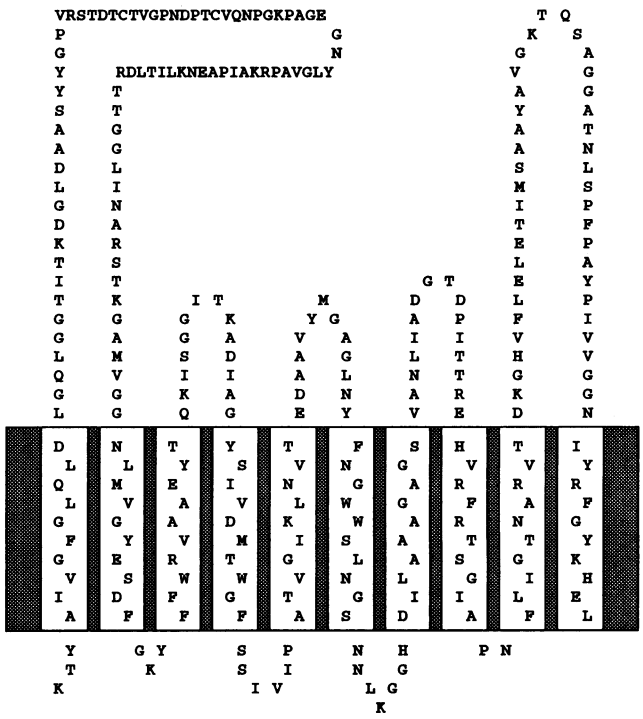

FIG. 5. Suggested topological model of OmpL1, containing 10 transmembrane segments in an alternating hydrophobic amino acid pattern. The transmembrane segments are shown in a staggered array with the hydrophobic, membrane-facing residues on the right side of the array. Some of the transmembrane segments are reflected as peaks on the beta-moment plot (Fig. 4B). The five surface-exposed loops are of various lengths and contain segments of high surface probability. The four periplasmic loops are typical of OMPs in that they are short and contain amino acids, such as proline (P), glycine (G), serine (S), and asparagine (N), which are turn promoters.

serum. Triton X-114 extraction of *L. alstoni* was carried out as described previously, in which it was demonstrated that Triton X-114 selectively solubilizes the leptospiral outer membrane (12). Immunoblots of *L. alstoni* fractionated with Triton X-114 revealed reactivity with the 31-kDa OmpL1 antigen in the whole organism and detergent-phase fraction but not the aqueous-phase fraction (Fig. 7). A characteristic of integral membrane proteins is that they selectively partition into the Triton X-114 detergent phase (5). Some OmpL1 reactivity was also found in the insoluble pellet. The antiserum was specific for OmpL1, making it a useful reagent for immunoelectron microscopy. Whole-mount immunoelectron microscopy with anti-OmpL1 antiserum demonstrated OmpL1 on the surface of *L. alstoni* (Fig. 8). Thirty-two organisms incubated in preimmune serum and 29 organisms incubated in anti-OmpL1 serum were quantitated for surface colloidal gold binding. Organisms incubated in preimmune serum had a mean of 26.1 particles bound per organism compared with 77.3 particles bound per organism incubated in anti-OmpL1 antiserum ($P < 0.0001$). The limited amount of colloidal gold particle binding was anticipated because of the low density of OMPs observed in *L. alstoni* by freeze fracture electron microscopy (12). Immunoelectron microscopic localization to the surface of *L. alstoni* provides ultrastructural evidence in support of the hypothesis that OmpL1 is an OMP.

DISCUSSION

The *ompL1* gene of *L. alstoni* serovar grippotyphosa encodes a protein with a predicted amino acid sequence consistent with that of an OMP. Transmembrane OMPs of gram-negative bacteria have a leader sequence with a leader peptidase I cleavage site which allows export through the cytoplasmic membrane. OmpL1 has a typical procaryotic leader peptide and leader peptidase I cleavage site (42). In this respect, OmpL1 conforms to the general finding that exported proteins of spirochetes have *E. coli*-like leader peptides and leader peptidase I cleavage sites (3, 6, 15, 33). The location of the OmpL1 leader peptidase I cleavage site is confirmed by the N-terminal amino acid sequence of the mature protein. The sequence leucine-serine-alanine which precedes the leader peptidase I cleavage site of OmpL1 also occurs at the leader peptidase I cleavage site of an exported plasmid protein (EppA) of *Borrelia burgdorferi* (6).

Other than the leader peptide, OmpL1 has no long, alpha-helical, hydrophobic membrane-spanning domains as found in cytoplasmic membrane proteins. X-ray crystallography and spectroscopic studies have shown that unlike cytoplasmic membrane proteins, which have long, alpha-helical, hydrophobic membrane-spanning domains, OMPs from gram-negative bacteria span the outer membrane as amphipathic beta-sheets (41, 45). The beta-sheet is an extended conformation which can cross the membrane in as

TABLE 1. Carboxy-terminal 10 amino acid residues of bacterial OMPs[a]

Protein	Organism	Residue at position (from C terminus):									
		10	9	8	7	6	5	4	3	2	1
OmpL1	*Leptospira alstoni*	**I**	**Y**	**R**	**F**	**G**	**Y**	**K**	**H**	**E**	**L**
PhoE	*Escherichia coli*	**I**	V	A	V	**G**	M	T	Y	Q	**F**
OmpC	*Escherichia coli*	**I**	V	A	L	**G**	L	V	Y	Q	**F**
BtuB	*Escherichia coli*	E	**Y**	T	L	S	G	S	Y	T	**F**
OmpT	*Escherichia coli*	F	I	T	A	**G**	L	**K**	Y	T	**F**
VirG	*Shigella flexneri*	Q	G	I	L	**G**	V	**K**	Y	T	**F**
Class I protein	*Neisseria meningitidis*	A	A	S	V	**G**	L	R	**H**	K	**F**
Class II protein	*Neisseria meningitidis*	A	S	M	V	**G**	L	R	**H**	K	**F**
PIA	*Neisseria gonorrhoeae*	V	G	G	V	**G**	L	R	**H**	K	**F**
PIB	*Neisseria gonorrhoeae*	A	S	A	V	V	L	R	**H**	K	**F**
Porin	*Bordetella pertussis*	A	V	G	V	**G**	I	R	**H**	R	**F**
P.69	*Bordetella pertussis*	T	F	H	A	**G**	**Y**	R	Y	S	**W**
LamB	*Escherichia coli*	T	F	G	A	Q	M	E	I	**W**	**W**
OmpS	*Legionella pneumophila*	Y	I	G	L	K	**Y**	V	G	N	**V**

[a] The carboxy-terminal transmembrane segment of these OMPs consists of a 10-amino-acid sequence with an alternating pattern of hydrophobic residues at positions 1, 3, 5, 7, and 9. PhoE, OmpC, BtuB, OmpT, and VirG demonstrate the typical Y-X-F carboxy-terminal motif. OmpL1 is similar to the neisserial proteins and *B. pertussis* porin in that a histidine is present three amino acids from the carboxy terminus. P.69, LamB, and OmpS are examples of OMPs without a carboxy-terminal phenylalanine. Boldface letters indicate residues shared with OmpL1.

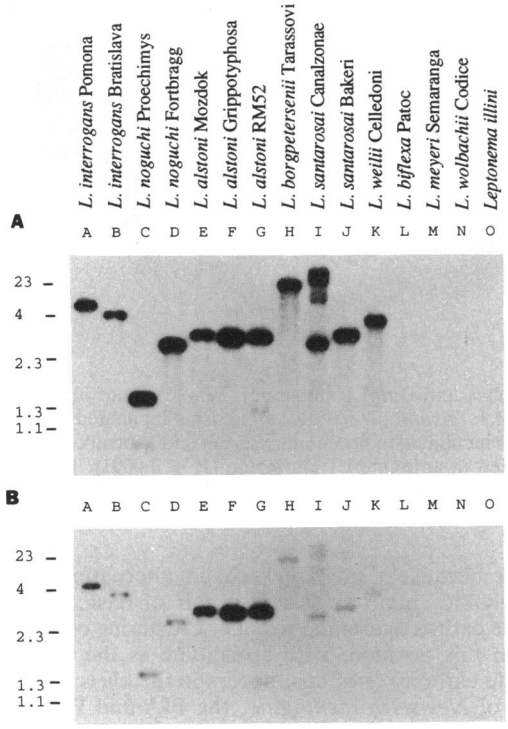

FIG. 6. Southern blot analysis of *Eco*RI-restricted genomic DNA from selected strains of *Leptospira*. The 834-bp *Nde*I-*Pvu*I *ompL1* gene fragment, which encodes 94% of the mature protein, was used to probe *Eco*RI genomic digests of 11 pathogenic and 4 nonpathogenic *Leptospira* strains. Panel A shows the results after washing at medium stringency in 2× SSC at 55°C. A single copy of the gene was present in all the tested strains of pathogenic *Leptospira* spp. The gene is not present in nonpathogenic strains of *Leptospira*. Panel B shows the results after washing at high stringency in 0.1× SSC at 55°C. The most homologous *ompL1* genes are found in other *L. alstoni* serovars. The higher-molecular-weight forms seen in the *L. santarosai* Canalzonai lane are due to incomplete *Eco*RI digestion (data not shown).

few as 10 amino acids (39). Because of the beta-sheet conformation, the OMP membrane-spanning segments are amphipathic because hydrophobic residues oriented towards the membrane alternate with more hydrophilic residues oriented towards the interior of the protein. Membrane-

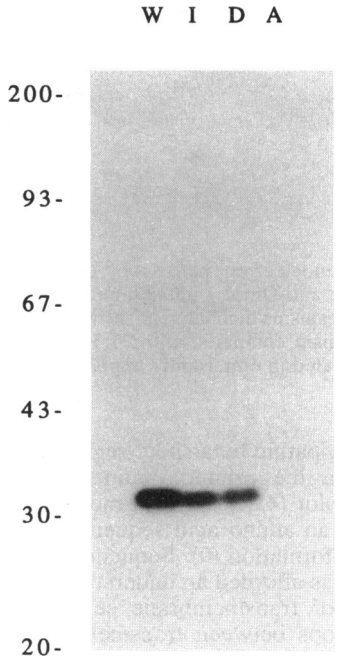

FIG. 7. Behavior of OmpL1 in Triton X-114 and specificity of anti-OmpL1 antiserum. An immunoblot of 10^8 culture-attenuated *L. alstoni* organisms probed with anti-OmpL1 antiserum is shown. Fractions analyzed were the whole organism (W) and Triton X-114-insoluble (I), detergent-phase (D), and aqueous-phase (A) material. The number of cell equivalents was the same in all four lanes. The locations of molecular size standards are shown (in kilodaltons) on the left. The 31-kDa OmpL1 protein partitions exclusively into the detergent phase, demonstrating the hydrophobic character of this protein, a feature shared by most transmembrane proteins. The antiserum is specific for OmpL1.

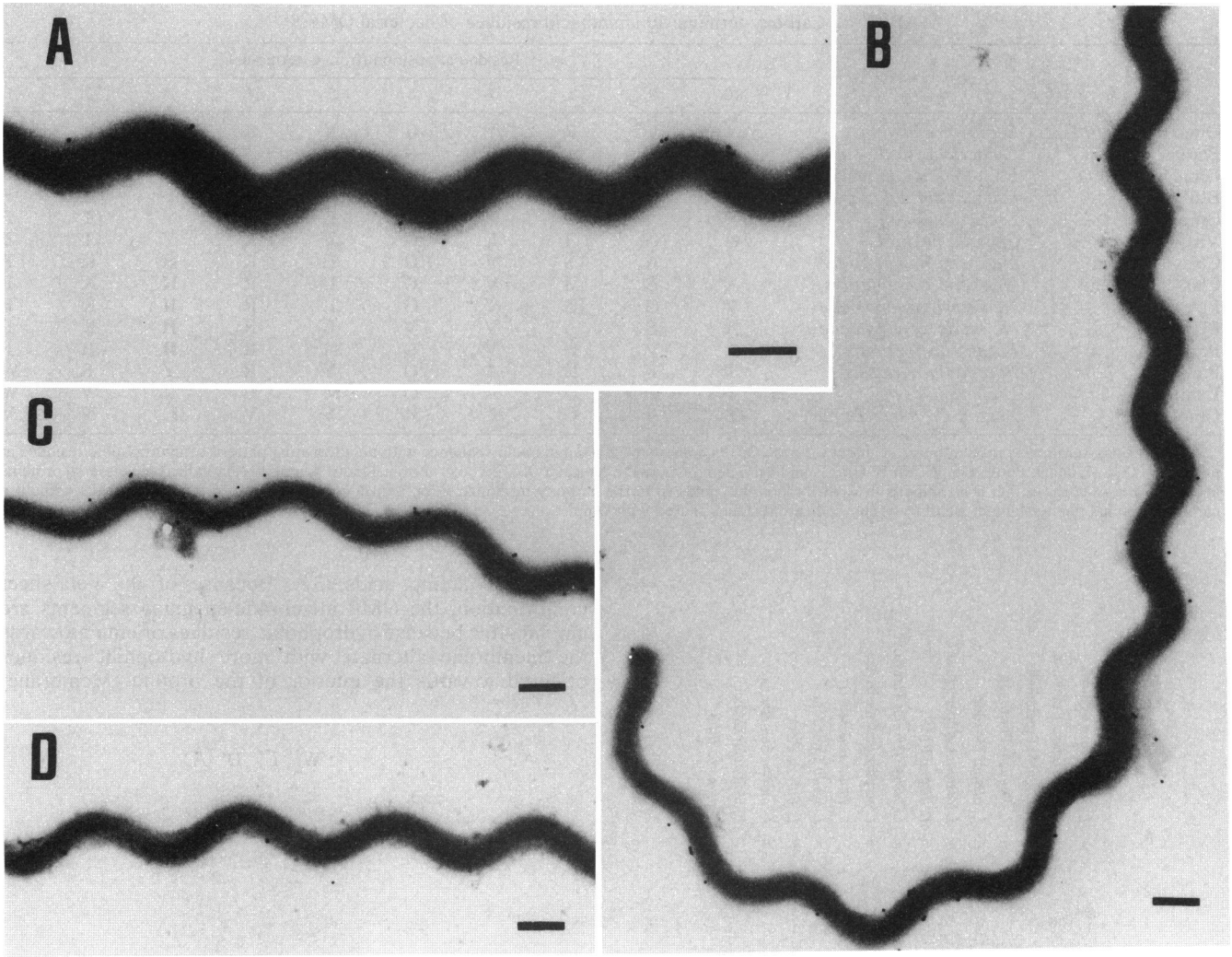

FIG. 8. Immunoelectron microscopy of whole *L. alstoni* serovar grippotyphosa anti-OmpL1 antiserum. Whole-mount immunoelectron microscopy with anti-OmpL1 antiserum demonstrates OmpL1 on the surface of *L. alstoni*. Thirty-two organisms incubated in preimmune serum and 29 organisms incubated in anti-OmpL1 serum were studied. Organisms incubated in preimmune serum had a mean of 26.1 particles bound per organism compared with 77.3 particles bound per organism incubated in anti-OmpL1 antiserum ($P < 0.0001$). Representative organisms incubated in either anti-OmpL1 antiserum (A, B, and C) or preimmune serum (D) are shown. Bars = 0.2 μm.

spanning amphipathic beta-sheet segments of OMPs can be predicted from the primary amino acid sequence by a beta-moment plot (41). The beta-moment is the calculated amphilicity of an amino acid sequence assumed to be in a beta-sheet conformation (9). Sequence alignment of homologous porins has afforded an understanding of the structural requirements of transmembrane segments and the short periplasmic loops between transmembrane segments (17). The OmpL1 sequence contains 10 stretches of amphipathic beta-sheet structure consistent with OMP transmembrane segments, making it possible to propose a topological model of OmpL1 (Fig. 5).

The transmembrane segments of homologous OMPs are conserved relative to surface-exposed regions (17). In particular, the carboxy-terminal transmembrane segment, which is generally formed by the carboxy-terminal 10 amino acids, has features which are conserved for OMPs from a broad range of gram-negative bacteria (35). The carboxy-terminal 10-amino-acid segment of OmpL1 is of special interest because there are both an alternating pattern of hydrophobic amino acids and a pair of basic amino acids near the carboxy-terminal residue. Clustering of basic amino acids at this position, with a histidine as the third residue from the carboxy terminus, occurs in the class 1 and class 2 porins of *Neisseria meningitis*, the PIA and PIB porins of *Neisseria gonorrhoeae*, and the *Bordetella pertussis* porin (17, 23, 25). The carboxy-terminal leucine of OmpL1 is somewhat atypical for an OMP in that a carboxy-terminal phenylalanine is lacking. However, as shown in Table 1, LamB of *E. coli*, P.69 of *B. pertussis*, and OmpS of *Legionella pneumophila* also represent variations on the carboxy-terminal phenylalanine motif (14, 35).

In addition to sequence analysis of OmpL1, two further lines of evidence presented in this study, obtained by using specific antiserum to the recombinant protein, support its outer membrane location. OmpL1 is partially solubilized by Triton X-114 extraction of *L. alstoni* and fractionates into the detergent phase. Our previous studies have demon-

strated the selective release of the leptospiral outer membrane by using the phase-separating, nonionic detergent Triton X-114 (12). In our previous study, we mentioned that preliminary data suggested that the 31-kDa protein was a component of the aqueous phase (12). However, the availability of specific antiserum has made it possible to determine unambiguously that OmpL1 partitions exclusively into the detergent phase. Fractionation of a protein into the hydrophobic detergent phase suggests that it is a membrane protein (5). Immunoelectron microscopy demonstrates specific binding to the surface of live organisms, indicating that OmpL1 has surface-exposed epitopes. Limited binding of colloidal gold particles to the surface is consistent with the low OMP density of *L. alstoni* as determined by freeze fracture electron microscopy (12).

The data presented in this study support the hypothesis that OmpL1 is an transmembrane OMP of pathogenic *Leptospira* spp. Further studies involving membrane fractionation, thin-section immunoelectron microscopy, and generation of liposomes containing purified OmpL1 are under way in an effort to test this hypothesis. OmpL1 would be the first spirochetal transmembrane OMP for which the structural gene has been cloned and sequenced, potentially allowing novel studies of the molecular basis of spirochetal OMP structure and function to be performed. The relationship of a transmembrane OMP, such as OmpL1, with the leptospiral outer membrane is likely to be different from that of lipoproteins OspA and OspB with the *B. burgdorferi* outer membrane. Transmembrane OMPs traverse the outer membrane and form particles that can be observed by freeze fracture electron microscopy, while lipoproteins are anchored to membranes only by their lipid moiety. Freeze fracture electron microscopy has demonstrated that the nonpathogen *Spirochaeta aurantia* has a high density of particles in its outer membrane (44), and a major outer membrane porin has been isolated from this spirochete (21). The increased expression of OmpL1 in culture-attenuated *L. alstoni* may suggest a porin function. The OMP-poor outer membrane of pathogenic spirochetes represents a diffusion barrier between the bacterium and its environment. Despite this diffusion barrier, *Leptospira* spp. are able to survive in the nutrient-poor environment outside the host, an important factor in the transmission of leptospirosis. Upregulation of porin expression following excretion from the host would represent an important adaptive response. Research on OmpL1 should also enhance our understanding of OMPs of other spirochetal pathogens such as *T. pallidum*, the causative agent of syphilis, and *B. burgdorferi*, the causative agent of Lyme borreliosis.

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ADDENDUM IN PROOF

In addition to the major outer membrane porin of *Spirochaeta aurantia* and OmpL1 of *Leptospira* spp., a major pore-forming 53-kDa surface antigen from the outer sheath of *Treponema denticola* has recently been described (C. Egli, W. K. Leung, K.-H. Müller, R. E. W. Hancock, and B. C. McBride, Infect. Immun. **61**:1694–1699, 1993).

REFERENCES

1. **Alexander, A. D., E. F. Lessel, L. B. Evans, E. Franck, and S. S. Green.** 1972. Preservation of leptospiras by liquid-nitrogen refrigeration. Int. J. Syst. Bacteriol. **22**:165–169.
2. **Bessen, D., and E. C. Gotschlich.** 1986. Interactions of gonococci with HeLa cells: attachment, detachment, replication, penetration, and the role of protein II. Infect. Immun. **54**:154–160.
3. **Blanco, D. R., M. Giladi, C. I. Champion, D. A. Haake, G. K. Chikami, J. N. Miller, and M. A. Lovett.** 1991. Identification of *Treponema pallidum* subspecies *pallidum* genes encoding signal peptides and membrane spanning sequences using a novel alkaline phosphatase expression vector. Mol. Microbiol. **5**:2405–2415.
4. **Blanco, D. R., E. M. Walker, D. A. Haake, C. I. Champion, J. N. Miller, and M. A. Lovett.** 1990. Complement activation limits the rate of *in vitro* treponemicidal activity and correlates with antibody-mediated aggregation of *Treponema pallidum* rare outer membrane protein. J. Immunol. **144**:1914–1921.
5. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:983–991.
6. **Champion, C. I., M. Giladi, D. A. Haake, D. R. Blanco, D. Foley, J. N. Miller, and M. A. Lovett.** Submitted for publication.
7. **Cunningham, T. M., J. N. Miller, and M. A. Lovett.** 1988. Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. J. Bacteriol. **170**:5789–5796.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis for the VAX. Nucleic Acids Res. **12**:387–395.
9. **Eisenberg, D., M. Wesson, and W. Wilcox.** 1989. Hydrophobic moments as tools for analyzing protein sequences and structures, p. 635–646. *In* G. D. Fasman (ed.), Prediction of protein structure and the principles of protein conformation. Plenum Publishing Corp., New York.
10. **Elkins, C., and P. F. Sparling.** 1990. Outer membrane proteins of *Neisseria gonorrhoeae*, p. 207–217. *In* E. M. Ayoub, G. H. Cassell, W. C. Branche, and T. J. Henry (ed.), Microbial determinants of virulence and host response. American Society for Microbiology, Washington, D.C.
11. **Everard, C. O. R., G. M. Fraser-Chanpong, and J. D. Everard.** 1987. The incidence of severe leptospirosis in Trinidad. Trop. Geogr. Med. **39**:126–132.
12. **Haake, D. A., E. M. Walker, D. R. Blanco, C. A. Bolin, J. N. Miller, and M. A. Lovett.** 1991. Changes in the surface of *Leptospira interrogans* serovar grippotyphosa during in vitro cultivation. Infect. Immun. **59**:1131–1140.
13. **Hansen, E. J., S. M. Robertson, P. A. Gulig, C. F. Frisch, and E. J. Hanes.** 1982. Immunoprotection of rats against *Haemophilus influenzae* type b disease mediated by monoclonal antibody against a *Haemophilus* outer membrane protein. Lancet **i**:366–368.
14. **Hoffman, P. S., M. Ripley, and R. Weeratna.** 1992. Cloning and nucleotide sequence of a gene (*ompS*) encoding the major outer membrane protein of *Legionella pneumophila*. J. Bacteriol. **174**:914–920.
15. **Isaacs, R. D., and J. D. Radolf.** 1990. Expression in *Escherichia coli* of the 37-kilodalton endoflagellar sheath protein of *Treponema pallidum* by use of the polymerase chain reaction and a T7 expression system. Infect. Immun. **58**:2025–2034.
16. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London)

317:262–264.
17. **Jeanteur, D., J. H. Lakey, and F. Patus.** 1991. The bacterial porin superfamily: sequence alignment and structure prediction. Mol. Microbiol. **5:**2153–2164.
18. **Jianguo, X., and D. Baomin.** 1989. Ultrastructure of leptospires after freeze-etching. J. West China Univ. Med. Sci. **20:**262–265.
19. **Johnson, R. C., and S. Faine.** 1984. Family II. *Leptospiraceae*, p. 62–67. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
20. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. **94:**27–31.
21. **Kropinski, A. M., T. R. Parr, B. L. Angus, R. E. W. Hancock, W. C. Ghiorse, and E. P. Greenberg.** 1987. Isolation of the outer membrane and characterization of the major outer membrane protein from *Spirochaeta aurantia*. J. Bacteriol. **169:**172–179.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Li, Z. M., J. H. Hannah, S. Stibitz, N. Y. Nguyen, C. R. Manclark, and M. J. Brennan.** 1991. Cloning and sequencing of the structural gene for the porin protein of *Bordetella pertussis*. Mol. Microbiol. **5:**1649–1656.
24. **Marck, C.** 1988. DNA Strider: a "C" program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16:**1829–1836.
25. **McGuinness, B., A. K. Barlow, I. N. Clarke, J. E. Farley, A. Anilionis, J. T. Poolman, and J. E. Heckels.** 1990. Comparative sequence analysis of the class 1 protein gene (porA) from three strains of *Neisseria meningitidis*: synthetic peptides define the epitopes responsible for serosubtype specificity. J. Exp. Med. **171:**1871–1882.
26. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
27. **Murphy, T. F., and L. C. Bartos.** 1988. Human bactericidal antibody response to outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infect. Immun. **56:**2673–2679.
28. **Nikaido, H.** 1988. Structure and functions of the cell envelope of gram-negative bacteria. Rev. Infect. Dis. **10:**S279–S281.
29. **Radolf, J. D., M. V. Norgard, and W. W. Schulz.** 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. Proc. Natl. Acad. Sci. USA **86:**2051–2055.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sansonetti, P. J.** 1991. Genetic and molecular basis of epithelial cell invasion by *Shigella* species. Rev. Infect. Dis. **13:**S285–S292.
32. **Saukkonen, K., H. Abdillahi, J. T. Poolman, and M. Leinonen.** 1987. Protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of *Neisseria meningitidis* B:15:P1.16 in infant rat infection model: new prospects for vaccine development. Microb. Pathog. **3:**261–267.
33. **Segers, R. P. A. M., A. Van der Drift, A. De Nijs, P. Corcione, B. A. M. Van der Zeijst, and W. Gastra.** 1990. Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar hardjo. Infect. Immun. **58:**2177–2185.
34. **Stoebner, J. A., and S. M. Payne.** 1988. Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56:**2891–2895.
35. **Struyve, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. **218:**141–148.
36. **Takafuji, E. T., J. W. Kirkpatrick, R. N. Miller, J. J. Karwacki, P. W. Kelley, M. R. Gray, K. M. McNeill, H. L. Timboe, R. E. Kane, and J. L. Sanchez.** 1984. An efficacy trial of doxycycline chemoprophylaxis against leptospirosis. N. Engl. J. Med. **310:**497–500.
37. **Thiermann, A. B.** 1984. Leptospirosis: current developments and trends. J. Am. Vet. Med. Assoc. **184:**722–725.
38. **Thiermann, A. B., R. D. McClellan, and H. T. Hill.** 1984. Improved techniques for the isolation of leptospires from swine abortion cases. Annu. Proc. Am. Assoc. Vet. Lab. Diagn. **27:**233–244.
39. **Tommassen, J.** 1988. Biogenesis and membrane topology of outer membrane proteins in *Escherichia coli*, p. 351–373. *In* J. A. F. Op den Kamp (ed.), Membrane biogenesis. NATO ASI Series, vol. H16. Springer-Verlag KG, Berlin.
40. **Towbin, H. T., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
41. **Vogel, H., and F. Jahnig.** 1986. Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. J. Mol. Biol. **190:**191–199.
42. **Von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184:**99–105.
43. **Walker, E. M., L. A. Borenstein, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1991. Analysis of outer membrane ultrastructure of pathogenic *Treponema* and *Borrelia* species by freeze-fracture electron microscopy. J. Bacteriol. **173:**5585–5588.
44. **Walker, E. M., G. A. Zampighi, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1989. Demonstration of rare protein in the outer membrane of *Treponema pallidum* subsp. *pallidum* by freeze-fracture analysis. J. Bacteriol. **171:**5005–5011.
45. **Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz.** 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science **254:**1627–1630.
46. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82:**1585–1588.
47. **Yasuda, P. H., A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner.** 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. Int. J. Syst. Bacteriol. **37:**407–415.
48. **Yelton, D. B., and N. W. Charon.** 1984. Cloning of a gene required for tryptophan biosynthesis from *Leptospira biflexa* serovar patoc into *Escherichia coli*. Gene **28:**147–152.