

The Rare Outer Membrane Protein, OmpL1, of Pathogenic *Leptospira* Species Is a Heat-Modifiable Porin

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The outer membranes of invasive spirochetes contain unusually small amounts of transmembrane proteins. Pathogenic *Leptospira* species produce a rare 31-kDa surface protein, OmpL1, which has a deduced amino acid sequence predictive of multiple transmembrane β -strands. Studies were conducted to characterize the structure and function of this protein. Alkali, high-salt, and urea fractionation of leptospiral membranes demonstrated that OmpL1 is an integral membrane protein. The electrophoretic mobility of monomeric OmpL1 was modifiable by heat and reduction; complete denaturation of OmpL1 required prolonged boiling in sodium dodecyl sulfate (SDS), 8 M urea, and 2-mercaptoethanol. When solubilized in SDS at low temperature, a small proportion of OmpL1 exhibited an apparent molecular mass of approximately 90 kDa, indicating the existence of an SDS-unstable oligomer. OmpL1 dimers and trimers were demonstrated by nearest neighbor chemical cross-linking. In order to generate purified protein for functional studies, the *ompL1* gene was ligated into the pMMB66 expression plasmid under control of the *tac* promoter. Although expression in *Escherichia coli* was toxic, most of the OmpL1 produced was found in the outer membrane, as determined by subcellular fractionation. Purified recombinant OmpL1 was reconstituted into planar lipid bilayers, demonstrating an average single channel conductance of 1.1 nS, similar to the major porin activity of native leptospiral membranes. These findings indicate that OmpL1 spans the leptospiral outer membrane and functions as a porin.

Leptospirosis is an important, global human and veterinary health problem caused by pathogenic spirochetes belonging to the genus *Leptospira*. In humans, leptospirosis is a zoonosis which occurs as a result of direct or indirect exposure to infected livestock, pets, or wildlife. Pathogenic *Leptospira* species are widespread in nature, reflecting their ability to survive and multiply both in the environment and in the renal tubules of reservoir hosts. Leptospirosis causes abortion, stillbirth, lack of milk production, and chronic renal infection with urinary shedding in cattle and swine, resulting in millions of dollars of agricultural losses annually. Commercially available vaccines, which consist of heat- or formalin-killed leptospores, produce incomplete or only short-term immunity, requiring their administration annually or semi-annually. In the case of *L. interrogans* serovar hardjo, the common bovine pathogen in North America, vaccines prepared in this way are ineffective (10, 11). Thus there is an important need for development of an improved leptospiral vaccine.

Spirochetes and gram-negative bacteria have an outer membrane which serves as a permeability barrier, protecting them from harsh environmental conditions encountered both inside and outside the host. The outer membrane of pathogenic spirochetes, including *Leptospira* species, is unlike that of typical gram-negative bacteria in that the density of transmembrane outer membrane proteins (OMPs) is extremely low (8, 22, 29, 43, 53, 54). Some OMPs of gram-negative bacteria function as porins, allowing the diffusion of hydrophilic solutes across the outer membrane into the periplasm. The OMPs of invasive spirochetes are of interest in understanding the physiology of

these organisms, because porin scarcity may restrict the availability of nutrients. OMPs are also of interest because they are located at the cell surface where bacterial pathogens interact with the host (3, 6, 27, 44). OMPs have been shown to be targets of bactericidal antibody (17, 37, 45), and there is evidence that OMPs can elicit protective antibodies against disease (26, 35, 45). OMP scarcity may be a mechanism by which pathogenic spirochetes persist in the host by evading the host immune response (9, 14).

Identification of OMPs of invasive spirochetes has been extremely difficult, because of their scarcity and the fragility of the spirochetal outer membrane. OmpL1 was originally identified by surface immunoprecipitation studies comparing virulent and culture-attenuated *L. kirschneri* (22). A 31-kDa protein (OmpL1) was shown to be present in extremely small amounts which were correlated with the low outer membrane particle density seen by freeze-fracture electron microscopy (22). We recently reported the molecular cloning and sequencing of the *ompL1* gene and demonstrated that it is present in all pathogenic *Leptospira* species that have been studied and is absent in nonpathogens (21). OmpL1 has been localized to the leptospiral surface by immunoelectron microscopy; however, its functional role and its relationship to the outer membrane were not characterized (21).

The deduced amino acid sequence of OmpL1 shares several characteristics with transmembrane OMPs of gram-negative bacteria, including a leader peptide and leader peptidase I cleavage site, and multiple amphipathic segments, suggestive of transmembrane β -strands (21). The electrophoretic mobility of OmpL1 was found to be heat modifiable, consistent with the stability of a protein with predominant β -structure. However, attempts to purify the native protein for functional analysis were unsuccessful because of the very low level of leptospiral OmpL1 expression. In order to study the function of OmpL1,

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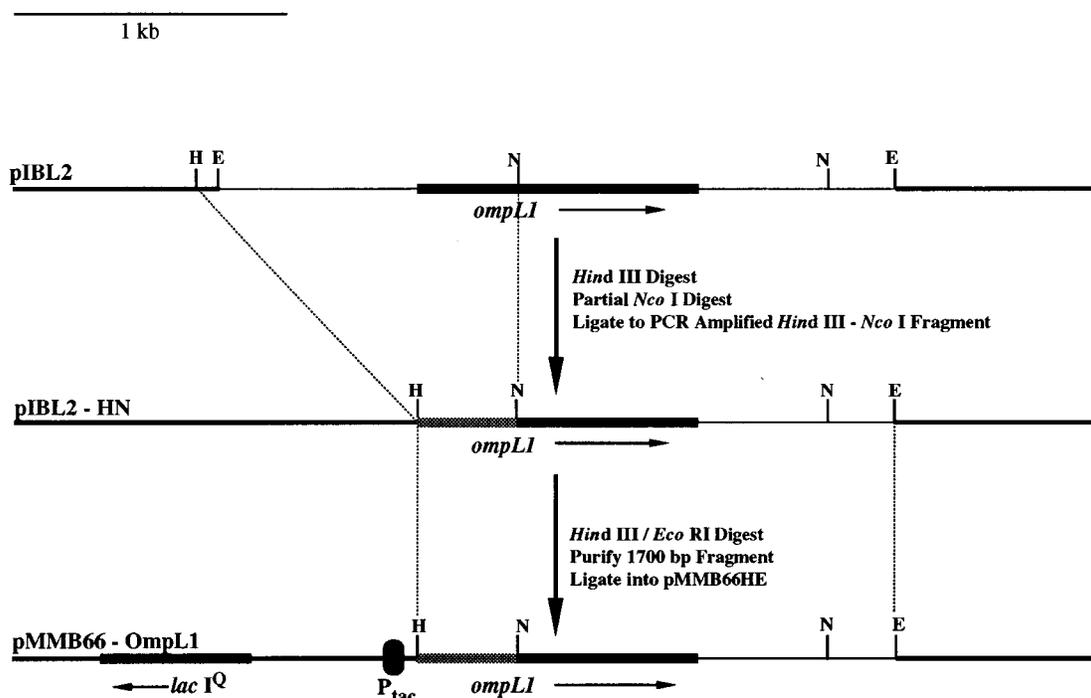


FIG. 1. Construction of the pMMB66-OmpL1 expression plasmid. The pIBL2 plasmid is the original pBluescript SK(-) isolate containing a 2.5-kb *EcoRI* fragment with the *ompL1* gene (21). PCR was used to amplify the *ompL1* gene with a *HindIII* restriction endonuclease site at its 5' end. The location of amplified DNA is indicated by shaded lines. Ligation into pMMB66HE placed expression of the *ompL1* gene under control of the *tac* promoter. Plasmid insert DNA (thin lines) is shown with part of the flanking pBluescript and pMMB66 vector DNA (thicker lines). The thickest lines indicate locations of genes. Single letters indicate the following restriction endonucleases: *EcoRI* (E), *HindIII* (H), and *NcoI* (N). See text for details.

its gene was cloned into the pMMB66 expression plasmid. OmpL1 expressed in *Escherichia coli* was localized, purified, and reconstituted in planar lipid bilayers. The results obtained provide direct evidence that OmpL1 functions as a porin in the outer membranes of pathogenic *Leptospira* species, the first demonstration of function for a recombinant spirochetal OMP.

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MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Culture-attenuated *L. kirschneri*, strain RM52 (formerly *L. alstoni*), was received from C. A. Bolin (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa) and passaged in bovine PLM-5 microbiological media (Intergen). *Escherichia coli* DH5 α [*supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] and *E. coli* CE1248 [*F*⁻ *recA56* *phoE* *proAB* *phoR69* *ompB471* (= *ompR*) *thr* *leu* *thi* *pyrF* *thy* *ilvA* *his* *lacY* *argG* *tonA* *rpsL* *cod* *dra* *utr* *glpR*] (52) were used for expression of intact OmpL1 encoded on the medium-copy-number expression vector pMMB66HE (gift of Michael M. Bagdasarian) (19). *E. coli* cells were routinely grown in Luria-Bertani (LB) broth or LB agar, unless otherwise mentioned (36). pIBL2 is the original pBluescript SK(-) plasmid isolate containing a 2.5-kb *EcoRI* fragment with the *ompL1* gene (21).

Gel electrophoresis and immunoblotting. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in final sample buffer (FSB) composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 8 M urea, unless otherwise noted. Proteins were separated on a 10% gel with a discontinuous buffer system (33) and transferred to nitrocellulose (Schleicher and Schuell) or polyvinylidene difluoride (Millipore) for immunoblotting with the Enhanced Chemiluminescence System (Amersham). Antiserum to OmpL1 was prepared as previously described

(21). Briefly, a New Zealand White male rabbit was immunized with purified His-6/OmpL1 fusion protein, expressed by *E. coli* JM109 (Invitrogen) transformed with the pRSET plasmid (Invitrogen) containing the *ompL1* gene (21). OmpL1 antiserum was used at a 1:5,000 dilution unless otherwise noted. Rabbit antisera to OmpA (gift of W. Wickner) and to *E. coli* leader peptidase 1 (gift of W. Wickner) were also used in this study. For two-dimensional gel electrophoresis, a 1.0-cm vertical lane was cut out of the first dimension gel and boiled in 2 ml of FSB for 30 min. The lane was then laid horizontally onto a second 10% gel and separated at 25 mA.

The pMMB66-OmpL1 expression plasmid. (i) **Cloning the *ompL1* gene in pMMB66HE.** Standard recombinant DNA procedures were performed as described previously (36). Restriction endonuclease digests were performed as recommended by the suppliers (New England Biolabs and Promega). The construction of the pMMB66-OmpL1 expression plasmid is shown in Fig. 1. Lacking a convenient restriction endonuclease site between the *ompL1* promoter and start codon, PCR was used to amplify the *ompL1* gene. Synthetic oligonucleotides were prepared by using an automated oligonucleotide synthesizer (380B, Applied Biosystems, Inc.). The 5' oligonucleotide contained the nucleotide sequence coding for the amino-terminal six amino acids of OmpL1 (21), including a *HindIII* restriction endonuclease site (underlined): 5'-AAG GAG AAG CTT ATG ATC CGT AAC ATA AGT-3'. The 3' oligonucleotide contained the nucleotide sequence coding for the carboxyl terminus of OmpL1, including an *EcoRI* restriction endonuclease site (underlined): 5'-TTG ATT GAA TTC TTA GAG TTC GTG TTT ATA-3'. The *ompL1* gene was amplified in a Programmable Thermal Controller (PTC-100, M. J. Research, Inc., Watertown, Mass.) with amplification for 30 cycles under standard conditions, using the above synthetic oligonucleotides and *Thermus aquaticus* polymerase (Promega). *L. kirschneri* genomic DNA, prepared as described previously (56), was used as template. The amplified *ompL1* gene was then ligated into pBluescript KS(+) after digestion with *HindIII* and *EcoRI*, transformed into *E. coli* DH5 α , and sequenced at the UCLA Core DNA Sequencing Facility by the dideoxy chain termination method with fluorescein-labeled dideoxy nucleotides (Applied Biosystems). A 378-bp *HindIII-NcoI* DNA fragment of the amplified, sequenced *ompL1* gene was then ligated into pIBL2, digested with *HindIII*, and partially digested with *NcoI*, so that only the *NcoI* site within the *ompL1* gene had been digested. The resultant plasmid, pIBL2-HN, was digested with *HindIII* and *EcoRI* in order to generate a 1,700-bp fragment containing the entire coding region of the *ompL1* gene. This 1,700-bp fragment was ligated into pMMB66HE, digested with *HindIII* and *EcoRI*. The resultant plasmid, pMMB66-*ompL1*, which places expression of OmpL1 under the control of the *tac* promoter, was used for transformation into *E. coli* strains DH5 α and CE1248.

(ii) **Viability of *E. coli* expressing OmpL1.** Separate cultures of *E. coli* DH5 α containing pMMB66HE with or without the *ompL1* insert were grown in SOB medium (36) supplemented with ampicillin (100 μ g/ml). At an optical density at 600 nm of 0.3, cultures were split and 1 mM isopropylthio- β -D-galactoside (IPTG, Sigma) was added to half of each culture. Samples were withdrawn prior to induction (time 0) and at subsequent intervals and serially diluted in phosphate-buffered saline, pH 7.4 (PBS). Ten microliters of each dilution was then plated onto LB plates supplemented with ampicillin, and the plates were incubated overnight at 37°C. Colonies were counted and CFUs were calculated from these dilutions.

Cell fractionation studies. (i) Leptospiral membrane fractionation. A leptospiral culture containing 1.5×10^{10} bacteria was washed twice in 5 mM MgCl₂-100 mM PBS at 4°C and resuspended in 1 ml of lysis buffer. Lysis buffer consisted of 10 mM Tris (pH 8), 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mg of lysozyme per ml. The bacterial suspension was incubated for 5 min at 4°C and subjected to three cycles of freezing (dry ice-ethanol) and thawing (with vigorous vortexing at room temperature). DNase was added, and the suspension was incubated for 20 min at 4°C, followed by centrifugation at top speed in a microcentrifuge at 4°C for 15 min. The supernatant was removed, and the membrane pellet was resuspended in 600 μ l of lysis buffer. A 100- μ l portion of the membrane fraction was diluted with 100 μ l of either cold 1.0% Triton X-100, 0.2 M Na₂CO₃, 3.2 M urea, 1.2 M NaCl, 0.2 N NaOH, or lysis buffer, followed by thorough mixing by pipetting and incubation for 15 min at 4°C. The samples were centrifuged at top speed in a microcentrifuge at 4°C for 15 min. Supernatants were carefully removed and acetone precipitated, and the precipitate was resuspended in FSB.

(ii) **Fractionation of *E. coli* expressing OmpL1.** Soluble (cytoplasm and periplasm), inner membrane, and outer membrane fractions were obtained by the method of Schnaitman (46). Overnight cultures of *E. coli* DH5 α (pMMB66-OmpL1) were inoculated into SOB medium supplemented with ampicillin and grown at 37°C until the cultures reached an optical density at 600 nm of 0.3. OmpL1 expression was then induced by adding IPTG to a final concentration of 0.5 mM. Following 1 h of incubation at 37°C the cells were collected by centrifugation and resuspended in 1.5 ml of 10 mM Tris, pH 8.0. The bacterial cells were burst open by passing through a French press (10,000 lb/in²) three times. Unbroken cells were removed by centrifugation at $5,000 \times g$. The cell extracts were separated into soluble and membrane fractions by centrifugation at 4°C at $15,000 \times g$ for 30 min. The supernatant, containing the cytoplasm and periplasm, was removed and concentrated by acetone precipitation. The membrane fraction was resuspended in 1 ml of 10 mM Tris, pH 8.0, containing 2% Triton X-100, tumbled overnight at room temperature, and then centrifuged at $15,000 \times g$ for 30 min. The supernatant was removed, and the Triton-soluble proteins were concentrated by acetone precipitation. The Triton-insoluble pellet was washed once in 1 ml of 10 mM Tris, pH 8.0.

Chemical cross-linking. A leptospiral culture containing 1.0×10^9 bacteria was washed three times in PBS and resuspended in 2.0 ml of PBS. An appropriate volume of 120 mM Bis (sulfosuccinimidyl) suberate (BS3) (Pierce Chemical Co.) and/or PBS was added to yield a final concentration of 0, 0.5, or 1.0 mM BS3 in a final volume of 4.8 ml. After rotation at room temperature for 2.5 h, the same amount of BS3 was added to each tube, followed by rotation for another 2.5 h. The reaction was quenched by the addition of 420 μ l of 50 mM ethanolamine-50 mM Tris and incubated for 15 min. The organisms were centrifuged at $15,000 \times g$ and resuspended in 50 μ l of FSB, boiled for 30 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with antiserum to OmpL1 at a dilution of 1:2,000.

Gel purification and planar lipid bilayer analysis. OmpL1 was expressed in the *E. coli* CE1248 host strain in the same way as in *E. coli* DH5 α , with the following exceptions. Cultures were grown in media supplemented with 0.4% glucose, and expression was induced with 1.0 mM IPTG. The crude membrane fraction was solubilized at room temperature in FSB containing 0.2% SDS without 2-mercaptoethanol and subjected to SDS-PAGE. The OmpL1 monomer band was excised from the gel and eluted overnight with 0.1% Triton X-100-100 mM NaCl at 4°C. As a control, *E. coli* CE1248 containing pMMB66HE without the *ompL1* insert was treated identically. The content of the samples eluted from both the OmpL1 and control lanes was assessed by SDS-PAGE and silver staining (55). Single-channel conductance studies were performed as described previously (5). Samples exhibiting porin activity were restudied after heating to 95°C for 20 min. The planar lipid bilayer apparatus used in these experiments is routinely calibrated with purified OprP from *Pseudomonas aeruginosa* (5).

RESULTS

Membrane fractionation of *L. kirschneri*. Treatment of bacterial cells with lysozyme and freeze-thawing, followed by centrifugation, separates bacterial proteins into soluble (cytoplasmic and periplasmic) and membrane fractions (28). The membrane fraction includes proteins associated with either the cytoplasmic or outer membrane. Treatment of the membrane fraction with high pH, high salt, or urea are standard techniques for solubilizing peripheral membrane proteins while

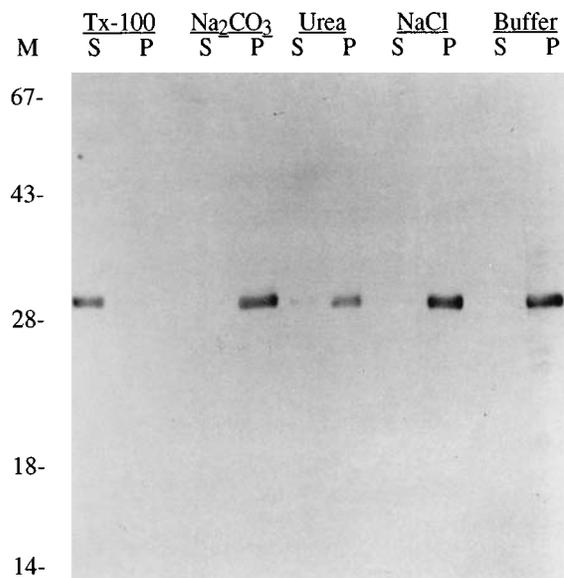


FIG. 2. Behavior of OmpL1 as an integral membrane protein. The *L. kirschneri* membrane fraction was diluted with lysis buffer as a control or adjusted to final concentrations of either 0.5% Triton X-100 (Tx-100), 0.1 M Na₂CO₃ (pH 11), 1.6 M urea, or 0.6 M NaCl. After 15 min of incubation on ice, all samples were separated into supernatant (S) and pellet (P) fractions by centrifugation, followed by SDS-PAGE and immunoblotting with anti-OmpL1 antisera. Molecular mass markers (M) are given in kilodaltons.

allowing integral membrane proteins to remain anchored to the lipid bilayer (18); whereas detergents, such as Triton X-100, are typically used to solubilize integral membrane proteins. This approach has been used to study both cytoplasmic (28) and outer (49) membrane proteins of *E. coli*. Immunoblot analysis of soluble and insoluble membrane fractions revealed that OmpL1 remained anchored to the insoluble membrane fraction when treated with 0.1 M Na₂CO₃, 1.2 M NaCl, or 3.2 M urea (Fig. 2). By comparison, when membranes were solubilized with 0.5% Triton X-100, OmpL1 was found in the soluble fraction. These results demonstrate that OmpL1 is an integral membrane protein.

Heat modification of OmpL1. The electrophoretic mobility of many outer membrane proteins of gram-negative bacteria is modified by heat. Two-dimensional gel electrophoresis was performed in order to assess if the electrophoretic mobility of OmpL1 was altered by more stringent denaturing conditions prior to separation in the second dimension. Proteins which are not heat modifiable migrate the same distance in both dimensions and appear on a diagonal. In contrast, the migration of heat-modifiable proteins is different in the second dimension resulting in their appearance at a location horizontally displaced off the diagonal. A sample of *L. kirschneri* was boiled for 5 min in FSB in order to obtain both the undenatured (25-kDa) and denatured (31-kDa) forms of OmpL1 in the first dimension (Fig. 3A). After complete denaturation by boiling for 30 min and separation in the second dimension, immunoblot analysis indicated the presence of the undenatured form off the diagonal (I) and the denatured form on the diagonal (II) (Fig. 3B). These results show that OmpL1 is a heat-modifiable protein and that the lower 25-kDa form is not a proteolytic breakdown product.

More detailed analysis was performed in order to assess the effect of reduction, heat, and urea on denaturation of OmpL1 (Fig. 4). Treatment of unheated samples with 2-mercaptoetha-

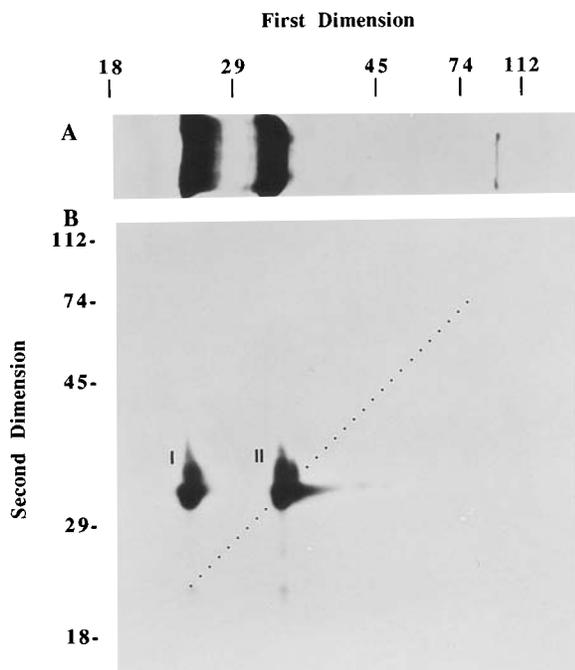


FIG. 3. Two-dimensional immunoblot showing heat modifiability of OmpL1. (A) In the first dimension, 5×10^8 *L. kirschneri* cells were washed in 2 ml of 5 mM MgCl₂-PBS, solubilized in 50 μ l of FSB containing 8 M urea at 100°C for 5 min, and separated by gel electrophoresis. (B) In the second dimension, a lane of the first gel was cut out and soaked in final sample buffer containing 8 M urea at 100°C for 30 min and separated by gel electrophoresis. After transfer, the blot was stained with amido black. Proteins which were not heat modifiable fell on the diagonal indicated by the dotted line. OmpL1 that was not completely denatured in the first dimension appeared at a location horizontally displaced from the diagonal (I). OmpL1 that was completely denatured in the first dimension appeared on the diagonal (II). Molecular mass markers are given in kilodaltons.

nol did not affect the mobility of the undenatured form of OmpL1 (apparent molecular mass of 25 kDa). Boiling for 10 min and even 30 min in FSB without urea resulted in only partial denaturation. Boiling with urea for 15 min resulted in a further loss of the 25-kDa form and the appearance of a 30- to 31-kDa doublet. Although relatively resistant to denaturation, OmpL1 could be completely denatured into a single 31-kDa form but required prolonged boiling in 8 M urea for 30 min. These results indicate that OmpL1 exists in a tightly folded conformation that requires rigorous treatment to completely unfold the protein and allow it to migrate at its predicted molecular mass.

Identification of OmpL1 oligomers. A heat-labile OmpL1 oligomer was detected by increasing the immunoblot sensitivity. When solubilized in SDS at low temperature, a small proportion of OmpL1 exhibited an apparent molecular mass of approximately 90 kDa, indicating the existence of an SDS-unstable oligomer (Fig. 5). Because of the low amount of oligomer present, this form of OmpL1 was not observed by two-dimensional gel electrophoresis (Fig. 3), suggesting that the underrepresentation of the oligomer on the immunoblot was not due to lack of antibody reactivity with the oligomer.

OmpL1 oligomers were also demonstrated with the membrane-impermeable, water-soluble chemical cross-linking reagent, BS3, which reacts with surface-exposed free amines located 11.4 Å (ca. 1.14 nm) apart. This bifunctional compound is useful in nearest neighbor analysis of the quaternary structure of oligomeric proteins. Intramolecular cross-linking of protein subunits occurs without intermolecular cross-linking

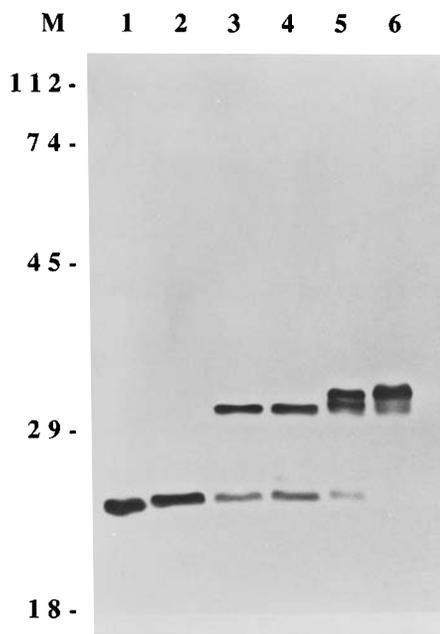


FIG. 4. Effects of heat, reduction, and urea on the electrophoretic mobility of monomeric OmpL1. Immunoblot analysis was performed using 10^8 *L. kirschneri* cells washed twice in 5 mM MgCl₂-PBS, solubilized in FSB under various conditions, and reacted with OmpL1 antiserum at a dilution of 1:5,000. Lanes: 1 and 2, 25°C, without urea, with and without reduction, respectively; 3 and 4, with reduction, without urea, 100°C for 10 and 30 min, respectively; 5 and 6, with reduction, with urea, 100°C for 10 and 30 min, respectively. Molecular mass markers (M) are given in kilodaltons.

of unrelated proteins. Treatment of *L. kirschneri* organisms with BS3 allowed the identification of OmpL1 dimers and trimers with apparent molecular masses of 65 and 106 kDa, respectively (Fig. 6). The heat-labile oligomer observed in Fig. 5 is probably a trimer, but it migrates more rapidly in the SDS-PAGE gel than the cross-linked trimer in Fig. 6. The difference in migration rate could be accounted for by two factors: firstly, the cross-linked trimer was denatured by boiling in FSB while the heat-labile oligomer was not; and secondly, the cross-linked trimer was modified by at least two molecules of BS3. Another difference between Fig. 5 and 6 is the requirement of a cross-linking reagent for the visualization of OmpL1 dimers.

Expression of OmpL1 in *E. coli*. Although expression of intact OmpL1 is toxic in *E. coli*, the pMMB66-*ompL1* construct was stable using this medium-copy-number expression plasmid. A 50-fold decrease in host cell viability was observed within 30 min of induction of the clone expressing OmpL1 (data not shown). Both induced and uninduced cultures containing only vector DNA, as well as uninduced bacteria containing the vector plus insert, continued to grow during this time period (data not shown). This toxic effect has also been observed with other heterologous porins expressed in *E. coli* (12, 31). The majority of OmpL1 expressed in *E. coli* DH5 α was found in the outer membrane fraction (Fig. 7). Outer membrane components were successfully separated from the cytoplasmic membrane as assessed by probing immunoblots using antisera to OmpA and leader peptidase as markers for the *E. coli* outer and cytoplasmic membranes, respectively. Consistent with previous observations (54a) using the anti-leader peptidase antiserum, we found a second, lower-molecular-mass band, below the 37-kDa leader peptidase band. Under these conditions, OmpL1 migrated as a doublet with the

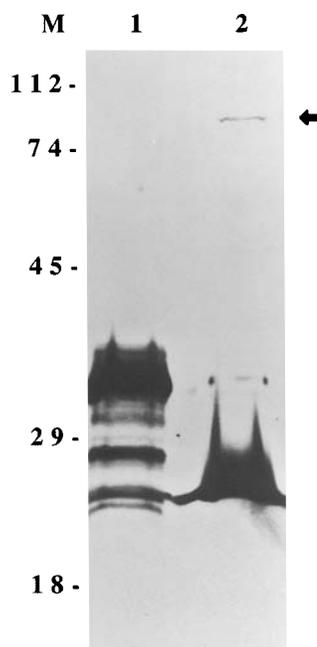


FIG. 5. Detection of a heat-labile, SDS-unstable OmpL1 oligomer. High-sensitivity immunoblot analysis was performed using OmpL1 antiserum at a dilution of 1:1,000 with overnight exposure of Kodak X-OMAT film. Lanes: 1 and 2, *L. kirschneri* solubilized in final sample buffer with urea for 30 min at 100 and 25°C, respectively. The location of the oligomeric form of OmpL1 is indicated by an arrow. Detection of multiple smaller-molecular-mass bands observed in lane 1 are due to the enhanced sensitivity of the immunoblot technique. Molecular mass markers (M) are given in kilodaltons.

upper band migrating in the same position as the native protein. The lower band may be due either to partial proteolysis or to unusual stability of a conformation of OmpL1 expressed in *E. coli*. When expressed in *E. coli*, the outer membrane protein CD of *Branhamella catarrhalis* also migrates as a doublet in

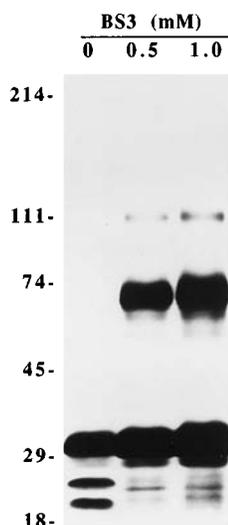


FIG. 6. Analysis of OmpL1 subunit structure using the nearest neighbor chemical cross-linking reagent, BS3. Whole *L. kirschneri* cells (10^8) were treated with 0, 0.5, or 1.0 mM BS3 prior to separation on an SDS-PAGE gel (4 to 12% polyacrylamide) and immunoblot analysis with antiserum to OmpL1. Molecular mass markers are given in kilodaltons.

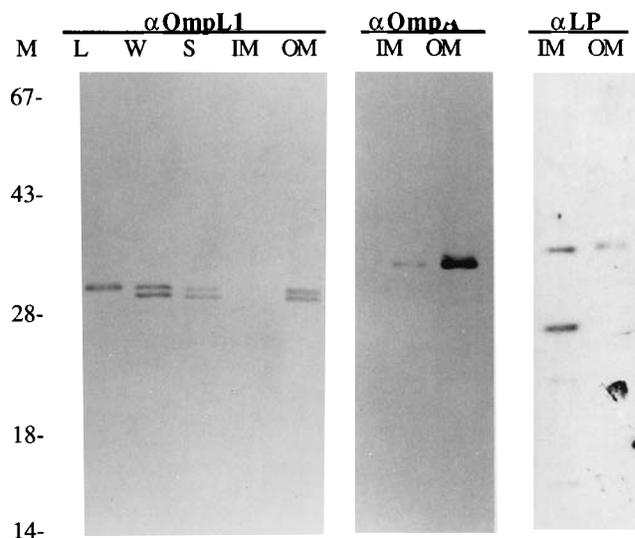


FIG. 7. Localization of OmpL1 expressed in *E. coli*. *E. coli* DH5 α bearing the pMMB66-OmpL1 plasmid was induced with 0.5 mM IPTG for 1 h and fractionated by the method of Schnaitman (46). *L. kirschneri* cells (L) were compared with whole *E. coli* cells (W) resuspended in final sample buffer or burst open by passing through a French press. The cell extracts were separated into soluble and membrane fractions by centrifugation at 4°C at $15,000 \times g$ for 30 min. The supernatant (S) contained the cytoplasm and periplasm. The membrane fraction was resuspended in 1 ml of 10 mM Tris, pH 8.0, containing 2% Triton X-100, tumbled overnight at room temperature, and then centrifuged at 4°C at $15,000 \times g$ for 30 min. The supernatant contained the inner membrane proteins (IM), and the pellet contained the outer membrane proteins (OM). Samples were subjected to SDS-PAGE and immunoblotting with anti-OmpL1, anti-OmpA, and anti-leader peptidase (LP) antiserum. Molecular mass markers (M) are given in kilodaltons.

SDS-PAGE, a property which has been attributed to two stable conformations of the protein (38).

Planar lipid bilayer assay. Recombinant OmpL1 was produced in the porin-deficient host strain *E. coli* CE1248. This strain has mutations preventing the production of porins OmpF, OmpC (because of the *ompB* mutation), and PhoE (52); LamB was repressed by growth in 0.4% glucose. The recombinant OmpL1 was separated from OmpA by SDS-PAGE after solubilization in 0.2% SDS without 2-mercaptoethanol (Fig. 8). The average single-channel conductance of gel-purified OmpL1 in 1 M KCl bathed lipid membrane was 1.1 nS (Fig. 9). This activity is similar to that observed after addition of a 1% Triton X-100 extract of *L. kirschneri* (Fig. 9). The porin activity of the gel-purified OmpL1 was lost after the sample was heated to 95°C for 20 min. Addition of the control sample derived from *E. coli* CE1248 containing pMMB66HE without the *ompL1* insert did not result in porin activity (data not shown).

DISCUSSION

In order to determine whether OmpL1 is an integral or a peripheral membrane protein, leptospiral membranes were fractionated by using a variety of reagents designed to solubilize peripheral membrane proteins by dissociating them from the lipid bilayer. OmpL1 remained membrane anchored despite treatment with alkaline pH, high salt, or urea. This approach has been used to evaluate eukaryotic (18) and *E. coli* integral (28, 49) membrane proteins. Most *E. coli* transmembrane OMPs, including OmpF and LamB, are alkali insoluble. OmpA is an exception to this pattern (28), perhaps as a result of its association with the peptidoglycan cell wall, which had

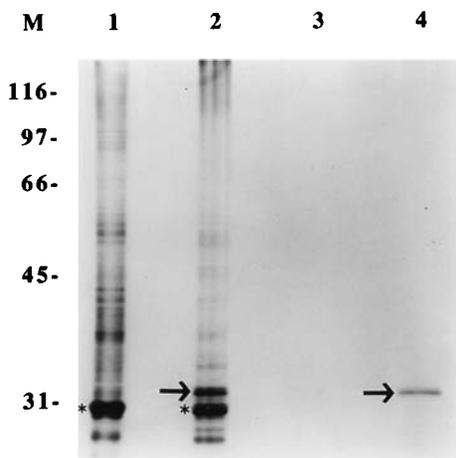


FIG. 8. Silver-stained SDS-PAGE gel showing purification of OmpL1. Crude membrane fractions from *E. coli* CE1248 containing the pMMB66 plasmid with and without the *ompL1* gene were solubilized at room temperature in 0.2% SDS without 2-mercaptoethanol and subjected to SDS-PAGE. Gel slices were removed from equivalent portions of both the OmpL1 and control lanes and eluted in 0.1% Triton X-100–100 mM NaCl. Lanes: 1 and 2, crude membrane fractions of *E. coli* CE1248 containing the plasmids pMMB66 and pMMB66-OmpL1, respectively; 3 and 4, eluates of gel slices obtained from the same material as loaded in lanes 1 and 2, respectively. Arrows show the location of OmpL1. Asterisks show the location of OmpA. Molecular mass markers (M) are given in kilodaltons.

been digested with lysozyme prior to exposure to alkali. Alkali fractionation does not differentiate between membrane-spanning proteins and lipoproteins, which are anchored to the lipid bilayer by fatty acids. The deduced amino acid sequence of OmpL1 suggests that it is not a lipoprotein. OmpL1 has a leader peptidase I cleavage site, indicating export beyond the inner membrane, whereas lipoproteins typically have leader peptidase II cleavage sites. In addition, the OmpL1 sequence has 10 predicted amphipathic membrane spanning domains, which are typically found in transmembrane OMPs (21).

A 31-kDa major cellular protein has been found to be associated with the outer membrane of *L. interrogans* serovar pomona (57). Two characteristics differentiate the 31-kDa major protein from OmpL1. The 31-kDa major protein is an abundant leptospiral protein, while OmpL1 is a rare leptospiral protein, consistent with quantitation of transmembrane OMPs by freeze-fracture electron microscopy (22). Secondly, the 31-kDa major protein is heat labile after extraction in Triton X-114 and incubation at 37°C, conditions under which OmpL1 has been shown to be heat stable (21).

Immunoblot analysis of two-dimensional gels showed that the more rapidly migrating forms of OmpL1 observed under less stringent conditions are a result of incomplete denaturation, not proteolytic breakdown. Treatment with 8 M urea was necessary for complete denaturation of OmpL1, a property of other OMPs including the *Yersinia enterocolitica* Ail protein (3). A decrease in electrophoretic mobility is observed with reduction (data not shown), suggesting that OmpL1 has an intrachain disulfide bond between cysteine 65 and cysteine 74. Intrachain disulfide bonds have been described in several other OMPs (24, 34, 38). These data show that OmpL1 has an OmpA-like pattern of heat-modifiable electrophoretic mobility, with slower migration occurring with more complete denaturation (4). Many porins retain their trimeric structure when solubilized in SDS at low temperature, exhibiting an OmpF-like pattern of heat-modifiable electrophoretic mobility. However, several SDS-unstable porin trimers have been described,

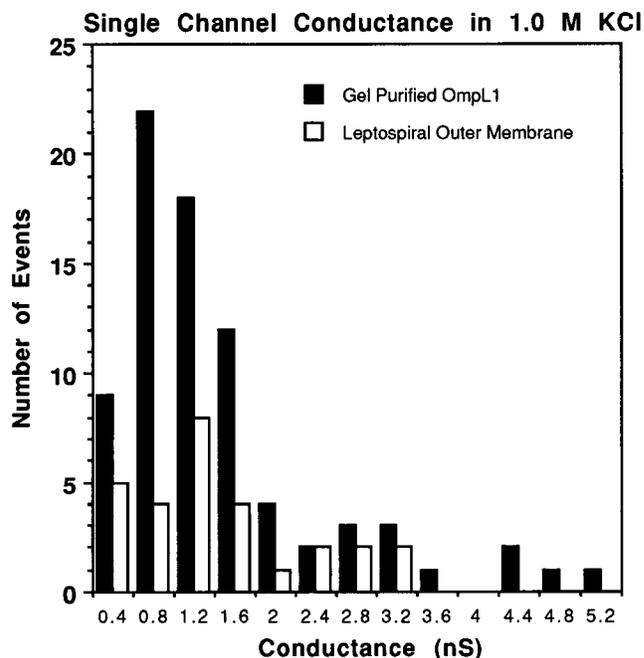


FIG. 9. Histogram of the single-channel conductance increases observed for the gel-purified recombinant OmpL1 (solid bars) and the Triton X-100-solubilized leptospiral membrane preparation (open bars) after addition to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. Leptospiral membrane was obtained by extraction of *L. kirschneri* in 1.0% Triton X-100.

including *Pseudomonas aeruginosa* OprF (2), the *Campylobacter jejuni* porin (41), and the porin of *Haemophilus influenzae* (51). Porin dimers and trimers of *H. influenzae* could only be demonstrated by nearest neighbor chemical cross-linking (30, 51). OmpL1 appears to exist in the leptospiral outer membrane as a trimer, based upon high-sensitivity immunoblot analysis (Fig. 5) and nearest neighbor chemical cross-linking experiments (Fig. 6). However, it should be noted that this result could be accounted for by heterogeneous OMP complexes, which have been described with *Neisseria gonorrhoeae* (39).

Demonstration that expression of the intact OmpL1 protein in *E. coli* resulted in outer membrane localization indicates recognition of the OmpL1 leader peptide and leader peptidase I cleavage site by the *E. coli* export pathway. These findings suggest that the OMP export pathway is conserved in these phylogenetically diverse bacteria. Overexpression in *E. coli* may result in artifactual localization of proteins. However, outer membrane localization of OmpL1 also occurred under conditions of very low level expression (data not shown). Localization to the outer membrane and not the periplasm indicates that OmpL1 possesses the targeting signals necessary for insertion into the *E. coli* outer membrane, although this could be accounted for by the ability of the mature OmpL1 protein to spontaneously fold into its native tertiary structure. While a specific outer membrane targeting signal is not known to exist (40), OmpL1 does have an amphipathic 10-amino-acid carboxy terminus, a feature which is conserved among many gram-negative OMPs (50).

Using the planar lipid bilayer assay, we have demonstrated that OmpL1 functions as a porin. The sensitivity of the planar lipid bilayer assay makes it necessary to exclude the possibility that trace contaminants are responsible for the observed porin activity. OmpL1 is produced in very small amounts in *L. kirsch-*

neri, rendering attempts to obtain highly purified native OmpL1 by gel filtration and cation-exchange chromatography, nondenaturing SDS-PAGE, and isoelectric focusing unsuccessful (data not shown). We were able to express and purify recombinant OmpL1 in *E. coli*, eliminating the possibility of contamination with other leptospiral proteins. Samples from *E. coli* CE1248 transformed with pMMB66 without the *ompL1* gene served as a negative control for contamination with *E. coli* porins. Although the production of recombinant porins may result in incorrect folding (48), several recombinant porins have been successfully expressed and refolded into their native conformation and shown to have porin activity identical to that of the native protein (13, 25, 42, 47).

The demonstration that OmpL1 functions as a porin confirms that it is a transmembrane OMP. These studies do not address the question of whether or not the channels are being formed by monomers or trimers in vivo. Additional model membrane experiments involving different salt solutions and salt concentrations may elucidate this. The average single-channel conductance for OmpL1 in 1 M KCl (1.1 nS) is within the range of known porins (23). By contrast, the 36.5-kDa porin of *Spirochaeta aurantia* and the 53-kDa porin of *Treponema denticola* have average single-channel conductances in 1 M KCl of 7.7 and 10.9 nS, respectively (16, 32), which are relatively large by gram-negative standards. The distribution of conductance measurements observed with the *L. kirschneri* outer membranes is similar to that of the *T. pallidum* outer membrane (8). The average single-channel conductance of the 31-kDa TROMP1 porin of *T. pallidum* is 0.7 nS, even smaller than that of OmpL1 (7). These data indicate that in these invasive spirochetes, diffusion of hydrophilic solutes across the permeability barrier of the outer membrane may be limited by the low density of OMPs. Larger channel diameters could have potentially compensated for porin scarcity, but that does not appear to be the case in either *T. pallidum* or pathogenic *Leptospira* species. Limitation of nutrient diffusion by porin scarcity is consistent with the slow metabolism and doubling times of pathogenic spirochetes. We have observed that as virulent *L. kirschneri* becomes culture attenuated, the decrease in doubling time is correlated with an increase in outer membrane particle density (20, 22). Determination of OmpL1 channel size would require additional electrical conductance measurements as well as alternative approaches, such as determination of the size exclusion limit by the liposome swelling assay.

Conservation of expression among pathogenic *Leptospira* species and its surface location make OmpL1 an attractive vaccine candidate. The low level of expression of OmpL1 in *Leptospira* spp. and its association with leptospiral LPS present technical barriers for purification of native protein. The demonstration that gel-purified recombinant OmpL1 had a single-channel conductance similar to that of the native protein implies a native conformation for the recombinant protein. The ability to produce large amounts of purified OmpL1 in its native conformation provides an important tool for immunoprotection studies in animal models of leptospirosis.

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