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A Newly Identified Leptospiral Adhesin Mediates Attachment to Laminin▽

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Pathogenic leptospires have the ability to survive and disseminate to multiple organs after penetrating the host. Several pathogens, including spirochetes, have been shown to express surface proteins that interact with the extracellular matrix (ECM). This adhesin-mediated binding process seems to be a crucial step in the colonization of host tissues. This study examined the interaction of putative leptospiral outer membrane proteins with laminin, collagen type I, collagen type IV, cellular fibronectin, and plasma fibronectin. Six predicted coding sequences selected from the *Leptospira interrogans* serovar Copenhageni genome were cloned, and proteins were expressed, purified by metal affinity chromatography, and characterized by circular dichroism spectroscopy. Their capacity to mediate attachment to ECM components was evaluated by binding assays. We have identified a leptospiral protein encoded by LIC12906, named Lsa24 (leptospiral surface adhesin; 24 kDa) that binds strongly to laminin. Attachment of Lsa24 to laminin was specific, dose dependent, and saturable. Laminin oxidation by sodium metaperiodate reduced the protein-laminin interaction in a concentration-dependent manner, indicating that laminin sugar moieties are crucial for this interaction. Triton X-114-solubilized extract of *L. interrogans* and phase partitioning showed that Lsa24 was exclusively in the detergent phase, indicating that it is a component of the leptospiral membrane. Moreover, Lsa24 partially inhibited leptospiral adherence to immobilized laminin. This newly identified membrane protein may play a role in mediating adhesion of *L. interrogans* to the host. To our knowledge, this is the first leptospiral adhesin with laminin-binding properties reported to date.

Leptospira interrogans, the causative agent of leptospirosis, is a highly invasive spirochete that efficiently colonizes target organs after penetrating the host, presumably via small abrasions or breaches of the surface integument (11). Leptospire invasiveness is attributed to its ability to multiply in blood, adhere to endothelial and epithelial cells, and penetrate into tissues. Protection against the host's innate immune mechanisms is achieved by its capacity to escape complement, notably the alternative pathway (25). Conversely, the nonpathogenic, saprophytic leptospires belonging to the species *Leptospira biflexa* are rapidly cleared from the bloodstream by phagocytosis (9, 43).

The mechanisms by which *L. interrogans* invades and colonizes the host are poorly understood, since very few virulence factors contributing to the pathogenesis of the disease have been identified (24). It has been well documented over the past decades that interaction of pathogens with the extracellular matrix (ECM) might play a primary role in the colonization of host tissues. The extracellular matrix of mammals is composed of two main classes of macromolecules: glycosaminoglycans (GAGs), usually found covalently linked to proteins in the form of proteoglycans, and fibrous proteins with both structural and adhesive functions, such as collagens, elastin, fibronectin, and laminin. Besides serving as a scaffold to stabilize the physical structure of tissues, the ECM plays an important role in regulating eukaryotic cell adhesion, differentiation, migration, proliferation, shape, and function. Under normal conditions, ECM is not exposed to bacteria. Pathogens may gain access to the ECM components after a tissue trauma following a mechanical or chemical injury or as a consequence of bacterial infection through the activity of toxins and lytic enzymes (23). Long-lasting infections may occur if microorganisms reach the subepithelial tissues.

Adherence to host tissues is mediated by surface-exposed proteins expressed by the microorganisms during infection. Among spirochetes, *Borrelia* spp. have been shown to express a 47-kDa outer membrane protein (OMP) named BBK32 that confers attachment to fibronectin (33, 34) as well as to the GAGs dermatan sulfate and heparin (12), two surface lipoproteins (DbpA and DbpB) that interact with decorin, a dermatan sulfate proteoglycan that is associated with and "decorates" collagen fibers (4, 16), and another GAG-binding adhesin termed Bgp (31, 32). *Treponema pallidum*, the causative agent of syphilis, also interacts with the ECM through outer surface proteins: Tp0155 and Tp0483 show specific attachment to fibronectin (6), and Tp0751 binds to a variety of laminin isoforms (5, 7). The only putative leptospiral adhesin identified to date is a 36-kDa fibronectin-binding protein isolated from the outer sheath of a virulent variant of pathogenic leptospires (26). A family of surface-exposed leptospiral proteins (lepto-

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spiral immunoglobulin-like, or *Lig*, proteins) with a structural organization that resembles that of bacterial adhesins has also been described (24, 30), but it remains to be confirmed whether the Lig proteins play a role in host cell attachment and invasion.

Whole-genome sequencing analysis of *L. interrogans* allowed identification of a repertoire of putative leptospiral surface proteins categorized as nonspecific porins, specific channels for nutrient acquisition, efflux channels, adhesins, S-layer glycoproteins, peripheral membrane proteins, and surface maintenance proteins (28). Of the 263 predicted genes encoding potential surface-exposed integral membrane proteins, 250 were previously unknown (29). Besides serving as targets for the host's immune system, it is possible that a number of proteins encoded by those genes mediate the initial adhesion to host cells.

In the present study, our goal was to identify *L. interrogans* ECM-binding proteins. For this purpose, five putative outer membrane proteins, selected via bioinformatics on the *L. interrogans* genome (28, 29, 37), and the previous characterized lipoprotein Loa22 (21) were expressed as recombinant proteins, and their capacity to mediate attachment to various ECM components was evaluated. We have identified and characterized a leptospiral membrane protein, named Lsa24 (leptospiral surface adhesin, 24 kDa) that binds strongly to laminin, a major adhesive glycoprotein of ECM and the basement membrane. Lsa24 was tested for the ability to competitively inhibit attachment of intact *L. interrogans* to laminin, and a moderate inhibition was achieved with in vitro experiments.

MATERIALS AND METHODS

ECM components. All macromolecules, including the control proteins fetuin and bovine serum albumin (BSA), were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was derived from human foreskin fibroblasts, plasma fibronectin was isolated from human plasma, and collagen type I was isolated from rat tail.

Leptospira strains and culture. A virulent strain of *L. interrogans* serovar Pomona and a culture-attenuated strain of *L. interrogans* serovar Copenhageni (Laboratório de Zoonoses, Faculdade de Medicina Veterinária e Zootecnia, Universidade São Paulo, São Paulo, Brazil) were used in the assays. The virulent strain is maintained by iterative passages in hamsters. Both strains were cultured at 29°C under aerobic conditions in liquid EMJH medium (Difco) with 10% rabbit serum enriched with L-asparagine, sodium pyruvate, calcium chloride, and magnesium chloride. For the ECM-binding assays, cells were harvested by centrifugation at 11,600 × *g* for 30 min and gently washed in sterile phosphate-buffered saline (PBS) twice. The leptospires were resuspended in PBS, and bacterial density was determined using dark-field microscopy and a Petroff-Hausser chamber.

In silico selection of outer membrane proteins. Cellular localization of the proteins was predicted by the PSORT program (http://psort.nibb.ac.jp) (27). Sequence motifs, including signal peptides, lipoprotein cleavage sites, and transmembrane domains, were searched for on public web servers (http://www.cbs.dtu.dk/services/SignalP) (20, 22) and (http://www.cbs.dtu.dk/services/TMHMM) (3).

Cloning, expression, and purification of recombinant proteins. Predicted coding sequences LIC10009, LIC10191, LIC10494, LIC11947, LIC12730, and LIC12906 were amplified by PCR from total *L. interrogans* genomic DNA (strain Fiocruz L1-130) using the primer pairs listed in Table 1. The Gateway (Invitrogen) system was used for the cloning and expression of proteins encoded by LIC10191, LIC10494, LIC11947, and LIC12730. PCR products were first cloned into a pENTR TOPO vector. Directional cloning was ensured by the addition of four bases to the forward primers (CACC) which annealed to a complementary overhang in the cloning vector (GTGG). DNA inserts were then transferred by recombination to the *Escherichia coli* expression vector pDEST17 using LR Clonase (Invitrogen). PCR fragments corresponding to LIC10009 and LIC12906 genes were cloned into the pGEM T-Easy vector (Promega) and transformed into *E. coli* DH5α. Following digestion with restriction enzymes (XhoI/HindIII for LIC10009 and XhoI/NcoI for LIC12906), fragments were subcloned into the *E. coli* expression vector pAE, which has been previously described (2, 36). All constructs were verified by DNA sequencing with appropriate vector-specific primers. Expression and purification of the resulting six-His-tagged recombinant proteins were performed as previously described (1). Protein refolding was achieved by multistep dialysis against 20 mM Tris-HCl (pH 8.0) and 0.5 M NaCl, gradually decreasing the urea concentration. Renatured proteins were extensively dialyzed against PBS for 24 to 48 h, and samples were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purified recombinant proteins were passed through columns containing immobilized polymyxin B (Detoxi-Gel AffinityPak prepacked columns; Pierce, Rockford, IL) for removal of residual *E. coli* lipopolysaccharide (LPS).

TABLE 1. Coding sequence-specific oligonucleotides used for DNA amplification and molecular masses of expressed recombinant proteins

Gene name[a]	Primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC10009	F, CGCGCTCGAGAAAAAGGAAGAA; R, AAGCTTTTATTGAAGAATAATTCC	26.5
LIC10191[b]	F, CACCGAGCCTTCAACGCAAGAGCAA; R, AACGTAAGACGTTGAGTTGCCACA	23.8
LIC10494	F, CACCACTGCTAGGGCTGCAGAAA; R, ACTTTGAGAGCTTCGTCTCGT	25.8
LIC11947[b]	F, CACCCCTTCGAGGTTGGAAATCG; R, AATCGATGGATCACGTTACG	22.8
LIC12730	F, CACCAGTTCTGACGGACTTCCCAA; R, TCTTGCGAATGAGTTGATCC	77.4
LIC12906	F, CTCGAGTATTCGTGTGGGGATAAA; R, CCATGGTAGAAATCAAACATCGCC	23.7

[a] *Leptospira interrogans* serovar Copenhageni (LIC) genes.
[b] Previously published by Gamberini and colleagues (14).

Circular dichroism spectroscopy. Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1-mm-path-length cell at 0.5-nm intervals. The spectra were presented as an average of five scans recorded from 190 to 260 nm.

Antisera against recombinant proteins. Ten female BALB/c mice (4 to 6 weeks old) were immunized subcutaneously with 10 μg of recombinant proteins. Aluminum hydroxide was used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same protein preparation. Negative control mice were injected with PBS. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers.

Binding of recombinant proteins to ECM. Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol (5) with some modifications. Briefly, ELISA plate wells (Nunc-Immuno plate, MaxiSorp surface) were coated with 1 μg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, BSA (nonglycosylated attachment-negative control protein), and fetuin (highly glycosylated attachment-negative control protein) in 100 μl of PBS for 2 h at 37°C. The wells were washed three times with PBS–0.05% Tween 20 (PBST) and then blocked with 200 μl of 1% BSA for 1 h at 37°C, followed by an overnight incubation at 4°C. One microgram of recombinant protein was added per well in 100 μl of PBS, and protein was allowed to attach to the different substrates for 1 h 30 min at 37°C. After washing six times with PBST, bound proteins were detected by adding an appropriate dilution of mouse antiserum in 100 μl of PBS. Dilutions of mouse antisera against each recombinant protein were as follows: LIC10009, 1:5,000; LIC10191 (Loa22), 1:10,000; LIC10494, 1:5,000; LIC11947, 1:2,000; LIC12730, 1:5,000; LIC12906 (Lsa24), 1:2,000. Incubation proceeded for 1 h, and after three washes with PBST, 100 μl of a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G in PBS was added per well for 1 h. All incubations took place at 37°C. The wells were washed three times, and *o*-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H_2O_2 was added. The reaction was allowed to proceed for 15 min and was then interrupted by the addition of 50 μl of 8 M H_2SO_4. The absorbance at 492

TABLE 2. Features, accession numbers, and probable locations of recombinant proteins

Gene	Protein accession no.	Feature	Probable location[b]
LIC10009	AAS68646	Lipoprotein probable	79% outer membrane, 70% inner membrane
LIC10191	AAS68819	Lipoprotein, OmpA-like (Loa22)[a]	93% periplasmic space, 25% outer membrane
LIC10494	AAS69115	Lipoprotein, probable	79% outer membrane, 70% inner membrane
LIC11947	AAS70529	Lipoprotein, probable	79% outer membrane, 70% inner membrane
LIC12730	AAS71287	Conserved hypothetical protein	93% outer membrane, 23% periplasmic space
LIC12906	AAS71459	Lipoprotein, probable (Lsa24)	28% inner membrane

[a] Characterized by Koizumi and Watanabe (21).
[b] Predicted by PSORT (27).

nm was determined in a microplate reader (Multiskan EX; Labsystems Uniscience). For determination of dose-dependent attachment of Lsa24 to laminin, protein concentrations varying from 0 to 1,000 nM in PBS were used. Binding was also evaluated as a function of varied laminin amounts from 0 to 1 μg. For statistical analyses, the attachment of Lsa24 to laminin was compared to its binding to BSA and to all ECM macromolecules by Student's two-tailed *t* test. A *P* value less than 0.05 was considered statistically significant.

Metaperiodate treatment of laminin. Microtiter wells were coated with 1 μg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with 50 mM sodium acetate buffer, pH 5.0, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 to 100 mM) in the same buffer for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 μl of 1% BSA for 1 h at 37°C. Binding of Lsa24 (1 μg in PBS per well) to periodate-treated laminin was assessed as outlined above.

***L. interrogans* ECM-binding assays.** Adhesion of intact *L. interrogans* to ECM components was performed according to a protocol described for spirochetes (33). Briefly, individual wells of Lab-Tek II chamber slides (Nalge Nunc International) were coated with 4 μg of laminin, cellular and plasma fibronectin, collagens I and IV, and the attachment negative control protein BSA, in PBS. After 16 h of incubation at 4°C, wells were washed with PBS and blocked for 2 h with 1% BSA in PBS. After washing the wells with PBS, 5×10^7 leptospires diluted in PBS were added to each well. Following a 2-h incubation at 29°C, wells were gently washed with PBS (six times for 5 min each time), and the attached spirochetes were visualized by dark-field microscopy. The number of leptospires in six 400× fields was enumerated, and the average number of bacteria per field was estimated.

Inhibition experiments. In vitro inhibition experiments were performed using the same protocol described for *L. interrogans* ECM-binding assays, except for the addition of 20 μg of the recombinant proteins Lsa24 and Loa22 (used as a negative control) to the blocking solution. Quantification of inhibition was performed essentially as described by Guo and colleagues (16). ELISA plate wells were coated with 1 μg of laminin, blocked and washed as already described, and then incubated with 0.5 μg of Lsa24 diluted in 100 μl PBS. Control wells were incubated with PBS. After washing with PBS, the wells were incubated with 50 μl of a suspension containing 2×10^8 spirochetes ml^{-1} PBS–0.1% BSA (PBSB) for 1 h at 37°C. Unattached organisms were removed after three washes with PBSB. The wells were then incubated for 1 h with 100 μl of a 1:10,000 dilution of anti-LipL32 serum in PBS. After three washes with PBSB, the wells were incubated for 1 h with 100 μl of a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G in PBS. All incubations were performed at 37°C. After washing, the reactions were developed as described above and the absorbance at 492 nm was taken.

Isolation of leptospiral OMPs by TX-114 extraction. OMPs were extracted according to a previously described method (18). In brief, leptospires cultured as outlined above were washed in PBS–5 mM $MgCl_2$ and then extracted in the presence of 2% Triton X-114 (TX-114; Sigma-Aldrich), 150 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA at 4°C for 2 h. The insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. After centrifugation, 20 mM $CaCl_2$ was added to the supernatant. Phase separation was performed by warming the supernatant at 37°C and subjecting it to centrifugation for 10 min at 1,000 × *g*. Three distinct fractions became apparent: the aqueous phase, the TX-114 phase, and the insoluble pellet. The detergent phase was precipitated with acetone. All fractions were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes for immunological analysis with Lsa24 antiserum.

Protein Data Bank accession numbers. The public database accession numbers for each protein sequence analyzed in this work are listed in Table 2. Proteins can also be accessed by their genome nomenclature for the gene locus and LIC numbers (*Leptospira interrogans* serovar Copenhageni).

RESULTS

Selection of putative surface proteins from genome sequences. With the ECM-binding studies in mind, our rationale for protein selection was mostly based on cellular localization, since surface proteins are potential targets for mediating adhesion to host. We focused on six proteins: four probable outer membrane proteins, according to genome annotation (29), one lipoprotein having a C-terminal OmpA consensus domain (17, 21, 37), and one protein annotated as hypothetical (29). Except for the previously characterized lipoprotein Loa22 (21), the proteins are new, of unknown function, and could be expressed and purified in our laboratory in concentrations and purities suitable to perform the binding assays. Gene locus identification, protein accession number, features, and computational predictions concerning cellular location of these proteins are listed in Table 2.

Expression, purification, and characterization of recombinant proteins. Oligonucleotides for PCR amplifications (Table 1) were designed from the genome, excluding the signal peptide sequences. The amplified coding sequences were cloned and expressed as full-length proteins in *E. coli*. Recombinant proteins were purified by metal chelation chromatography, and an aliquot of each protein was analyzed by SDS-PAGE (Fig. 1). As we can see from this figure, most of the contaminants were washed away and all proteins are represented as single major bands. Structural integrity of the purified proteins was assessed by CD spectroscopy. As depicted in Fig. 2, the minima at 208 and 222 nm and the maximum at 192 nm in the CD spectrum showed the high α-helical secondary structure con-

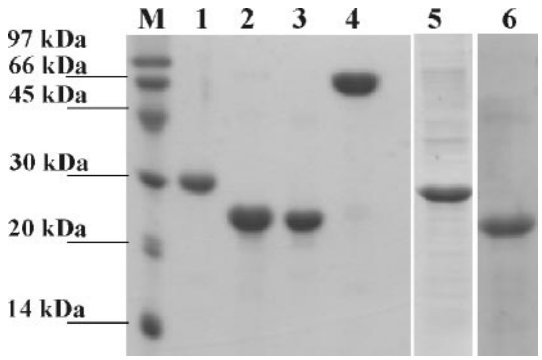

FIG. 1. Purification of recombinant proteins: SDS-PAGE (15%) of purified recombinant proteins obtained by metal affinity chromatography. Proteins encoded by LIC10009 (lane 1), LIC10191, known as protein Loa22 (lane 2), LIC11947 (lane 3), LIC12730 (lane 4), LIC10494 (lane 5), LIC12906, named Lsa24 (lane 6) are shown. Lane M, molecular mass standard.

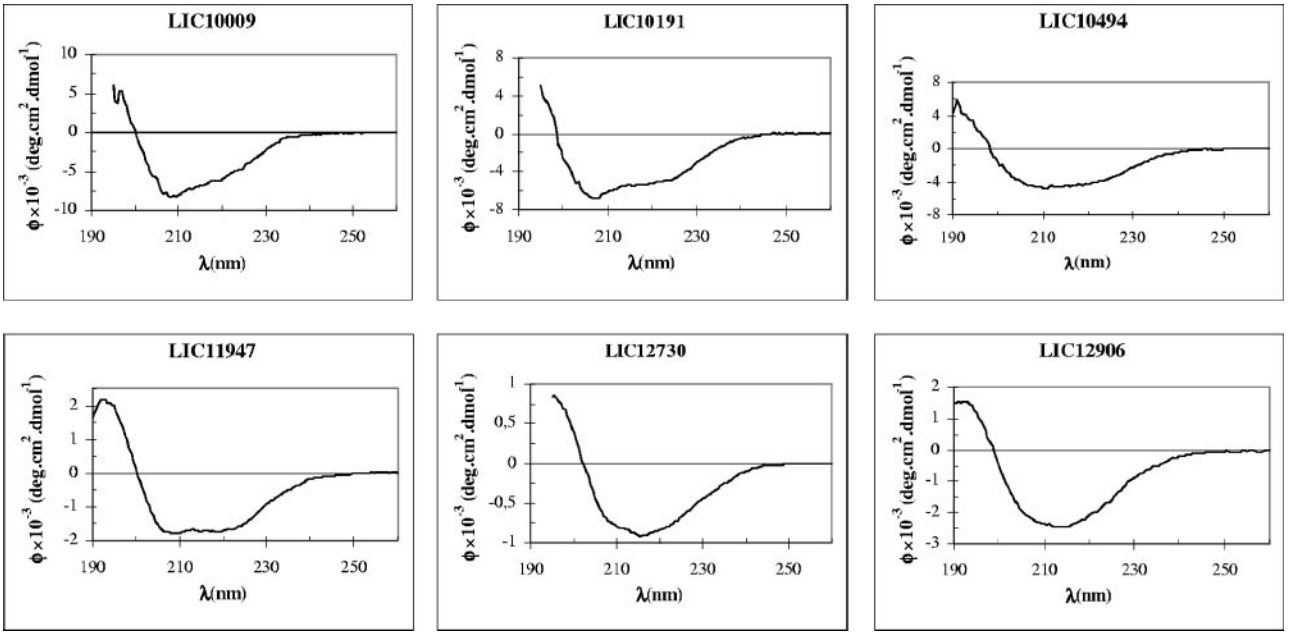

FIG. 2. Circular dichroism spectra of the recombinant proteins. CD spectra of proteins encoded by LIC10009, LIC10191 (Loa22), LIC11947 (predominant α-helical secondary structure), LIC10494 (both α-helical and β-strand), and LIC12730 and LIC12906 (Lsa24) (predominant signal of β-strand) are shown. Far-UV CD spectra are presented as an average of five scans recorded from 190 to 260 nm. ϕ, molar ellipticity.

tent of the recombinant protein encoded by LIC11947. LIC10009-encoded protein and Loa22 also seemed to have a predominance of α-helices, while the protein encoded by LIC10494 may have both α-helices and β-sheets in its secondary structure composition. LIC12730-encoded protein and Lsa24 presented a predominant signal of β-strands, with minimum ellipticities around 215 nm. The experimental data confirmed the secondary structure content previously predicted by computational analysis (http://bioinf.cs.ucl.ac.uk/psipred/).

Adhesion of recombinant proteins to ECM components. Laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, and the control proteins BSA and fetuin were immobilized on microdilution wells, and recombinant protein attachment was assessed by an ELISA-based method. As shown in Fig. 3, Lsa24 exhibited a significant level of attachment to laminin, 40-fold above the background level seen with immobilized BSA. Binding of Lsa24 to laminin was also significant compared to its binding to collagen IV ($P < 0.05$) and cellular fibronectin ($P < 0.05$) (Table 3). No binding was observed when wells were coated with collagen type I or with the highly glycosylated control protein fetuin. A moderate binding to collagen type I, collagen type IV, and plasma fibronectin was observed with Loa22 (Fig. 3), but optical density values registered at 492 nm were too low to prompt further investigation (Table 3). No specific binding to the target ECM macromolecules was detected with proteins encoded by LIC10009, LIC10494, LIC11947, and LIC12730 (Fig. 3). In fact, LIC10009 encoded protein bound to all coating macromolecules, including the control proteins BSA and fetuin. It seems that those interactions are more due to a general stickiness of the protein or, alternatively, due to residual contaminants not fully eliminated during the purification step rather than to specific interactions. Data are presented as the magnitude of attachment over the negative control protein BSA (arbitrarily set equal to 1) to emphasize specificity of attachment to the ECM macromolecules.

The interaction between Lsa24 and laminin was also assessed on a quantitative basis as illustrated in Fig. 4. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant protein (0 to 1,000 nM) were allowed to adhere to a fixed laminin amount (1 μg). The saturation level was reached at a protein concentration of 500 nM (Fig. 4A). Binding was also evaluated as a function of laminin concentration for a given recombinant protein concentration (1 μg). As shown in Fig. 4B, the degree of attachment increased proportionally as a function of laminin concentration and reached its maximum level at 1 μg of laminin. Increasing the laminin or protein concentration had no effect on the Loa22-laminin interaction (Fig. 4). This recombinant protein was included in our experiments as a negative control.

All recombinant proteins used in the binding assays presented above were treated with polymyxin B to ensure removal of residual *E. coli* LPS that could eventually interfere with the assays. In fact, we compared absorbance values before and after polymyxin treatment and found that Loa22, Lsa24, and the protein encoded by LIC11947 showed a partial reduction in their optical density values after treatment, suggesting a possible involvement of LPS in mediating attachment to ECM (data not shown).

Effects of laminin oxidation on protein binding. In order to address the role of laminin sugar moieties in the interaction with Lsa24, laminin was oxidized by increasing concentrations of sodium metaperiodate, ranging from 5 to 100 mM. The effect of periodate concentration on the binding is shown in Fig. 5. Laminin oxidation was performed at 4°C for 15 min. This mild treatment ensures cleavage of vicinal carbohydrate

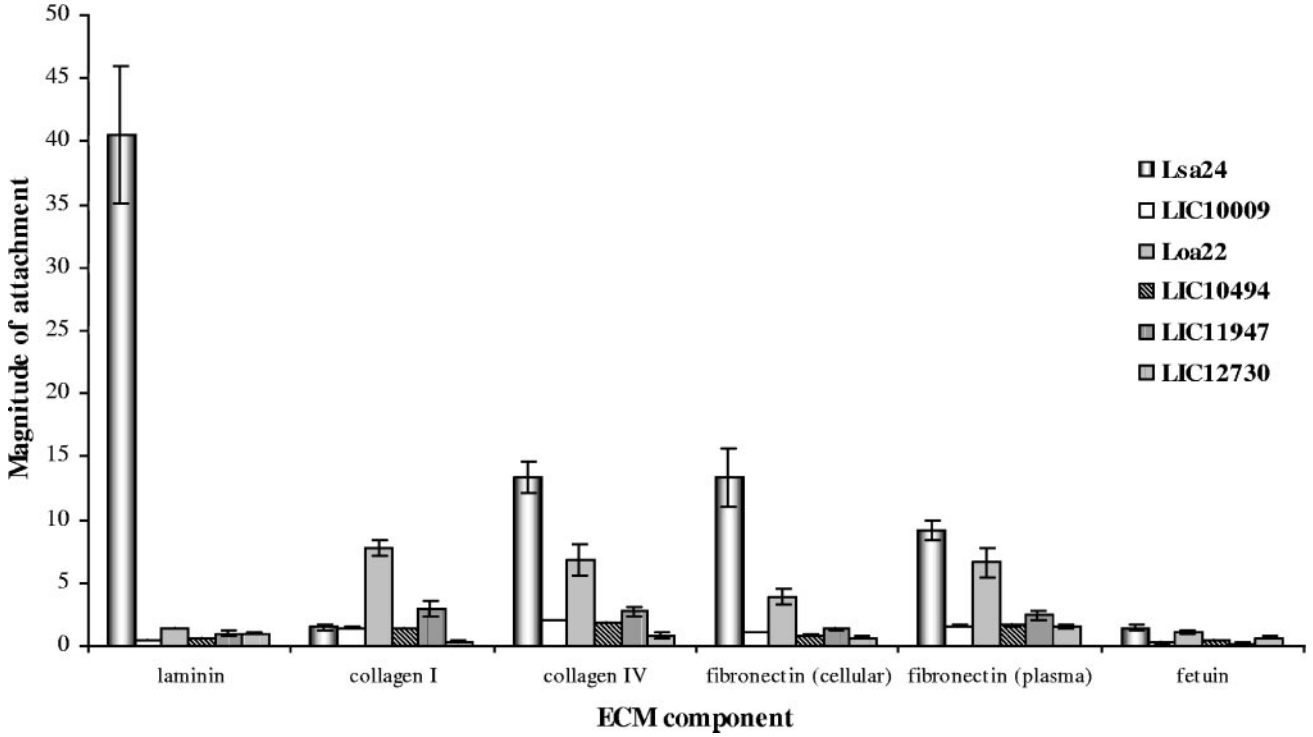

FIG. 3. Binding of recombinant proteins to ECM components. Wells were coated with 1 μg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, and the control proteins BSA and fetuin. Recombinant protein attachment to those ECM macromolecules was assessed by an ELISA-based assay. One microgram of recombinant protein was added per well. Optical densities were taken at 492 nm. Specific profiles of adhesion to the various coating macromolecules are shown as magnitude of attachment (fold) over the negative control protein BSA, arbitrarily set equal to 1. Data represent the mean ± standard error of three independent experiments.

hydroxyl groups while the polypeptide chain structure remains intact (44). The oxidation effect was dose dependent, and a considerable reduction (72%) in Lsa24 attachment to metaperiodate-treated laminin was observed at a 10 mM concentration of periodate (Fig. 5). These results indicate that laminin sugar residues are critical for the interaction of Lsa24 with this major ECM glycoprotein.

***L. interrogans* attachment to ECM macromolecules.** The ability of intact *L. interrogans* to adhere to various ECM proteins was evaluated by dark-field microscopy. Leptospires attached to chamber slides coated with laminin at an average density of 40 organisms per 400× field (Fig. 6A). Slides coated with collagen I, collagen IV, cellular fibronectin, or plasma fibronectin bound approximately 100 to 140 leptospires per 400× field, as shown in Fig. 6B to E, respectively. In contrast, control slides coated with BSA bound fewer than five organisms per 400× field (Fig. 6F).

Inhibition of *L. interrogans* attachment to laminin by Lsa24. The effect of recombinant Lsa24 on leptospiral adherence to laminin was first examined by in vitro inhibition experiments (Fig. 7). Laminin-coated chamber slides were preincubated with Lsa24 for 2 h before whole *L. interrogans* cells were added. Attached leptospires were visualized by dark-field microscopy. The addition of Lsa24 reduced leptospire binding to laminin-coated slides (Fig. 7B). Attachment was not affected by prior incubation with the negative control protein Loa22 (data not shown). The inhibitory effect exerted by Lsa24 on leptospiral adherence was quantified by an ELISA-like method. Laminin-coated microtiter wells were incubated with 0.5 μg of Lsa24 prior to the addition of 1×10^7 *L. interrogans*. Wells were

TABLE 3. Binding of Lsa24 and Loa22 to ECM components

Recombinant protein	Binding to ECM component[a]						
	Laminin	Collagen I	Collagen IV	Fibronectin		Fetuin	BSA
				Cellular	Plasma		
Lsa24	0.75 ± 0.028	0.03 ± 0.027	0.26 ± 0.183	0.24 ± 0.043	0.17 ± 0.063	0.03 ± 0.002	0.02 ± 0.010
Loa22	0.03 ± 0.021	0.17 ± 0.029	0.14 ± 0.062	0.08 ± 0.041	0.14 ± 0.064	0.03 ± 0.031	0.02 ± 0.011

[a] Wells were coated with 1 μg of each ECM component, and 1 μg of recombinant protein was added per well. Values are optical densities at 492 nm (95% level of confidence). The attachment of Lsa24 to laminin was compared to the attachment of the protein to BSA by the two-tailed *t* test ($P < 0.05$). Binding of Lsa24 to laminin was also significant compared to its binding to all ECM proteins mentioned above ($P < 0.05$).

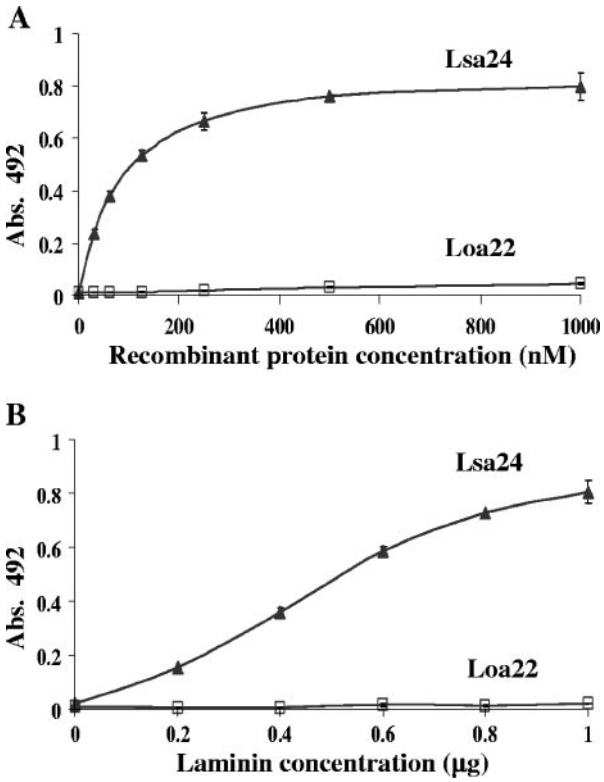

FIG. 4. Binding of Lsa24 to laminin as a function of protein and laminin concentration. (A) Binding of laminin (1 μg) with recombinant protein (concentration ranging from 0 to 1,000 nM); (B) recombinant protein (1 μg) with laminin (amount ranging from 0 to 1 μg). Each point represents the mean absorbance value at 492 nm ± the standard deviation of three independent experiments. Loa22 was included as a negative control since it showed no specific attachment to laminin.

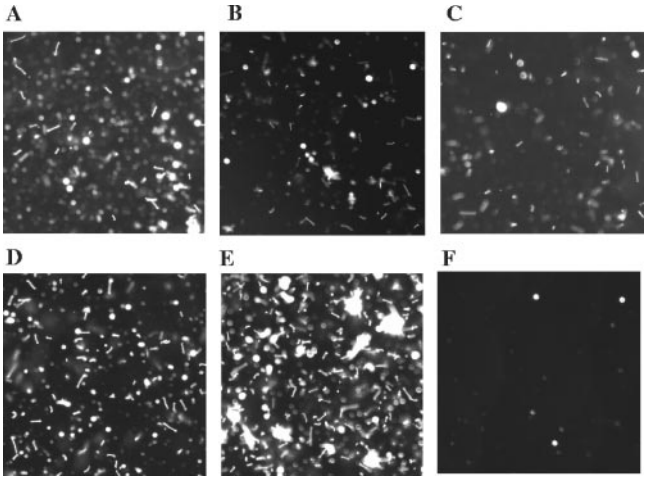

FIG. 6. *L. interrogans* attachment to ECM macromolecules. Glass slides coated with laminin (A), collagen I (B), collagen IV (C), cellular fibronectin (D), plasma fibronectin (E), or BSA (F) were incubated with 5×10^7 leptospires (*L. interrogans* serovar Pomona). Attachment was visualized using dark-field microscopy.

probed with anti-LipL32 serum, based on the fact that LipL32 is a major outer membrane leptospiral protein (18). Lsa24 caused a modest but significant reduction in the number of leptospires adhering to laminin ($P < 0.05$) (Fig. 7C). The experiment was repeated three times with similar results.

Cellular localization of Lsa24. To determine whether Lsa24 is localized at the membrane, we performed an extraction of the *L. interrogans* outer membrane with the nonionic detergent Triton X-114. The three fractions derived from TX-114-extracted material after incubation at 37°C were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with Lsa24 antiserum. Lsa24 was found to be completely absent from the detergent-insoluble pellet fraction but

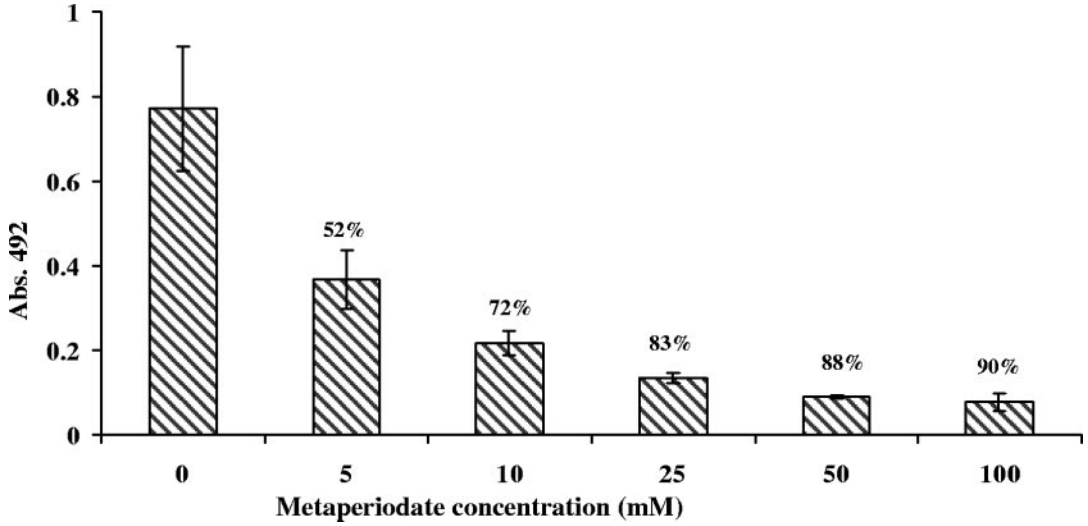

FIG. 5. Sugar moiety contribution to the laminin-Lsa24 interaction. Immobilized laminin was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark. Mean absorbance values at 492 nm (± the standard deviations of three independent experiments) were compared to those obtained with untreated laminin (0 mM). The percent reduction in protein attachment to metaperiodate-treated laminin as a function of periodate concentration is indicated above each bar.

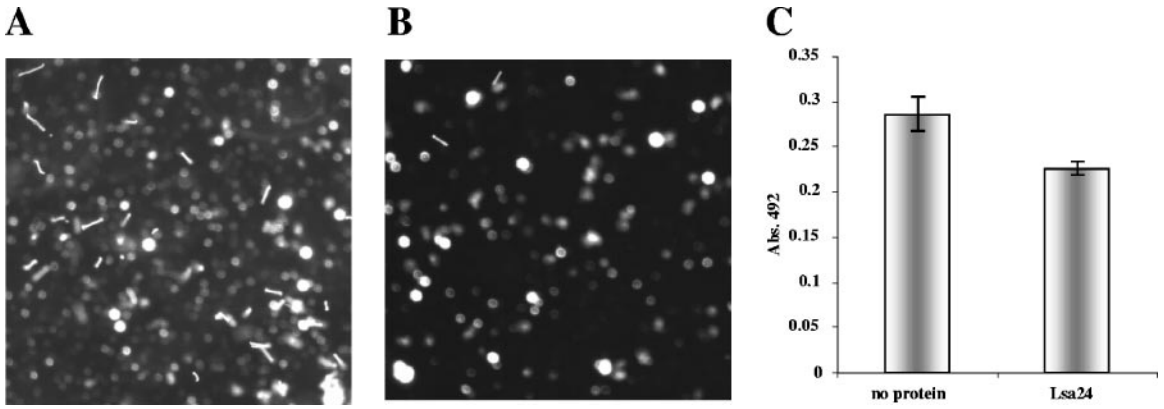

FIG. 7. Inhibition of *L. interrogans* attachment to laminin by Lsa24. Chamber slides were coated with 4 μg of laminin for 16 h. The slides were blocked with BSA, and 5×10^7 leptospires were added to each chamber. (A) No protein added to the blocking solution; (B) 20 μg of Lsa24 was added to the blocking solution to test for its ability to competitively inhibit attachment of *L. interrogans* to laminin. Adherence was visualized by dark-field microscopy. (C) *Leptospira* attachment to the substrate was quantified by ELISA. Laminin-coated microtiter wells were incubated with 0.5 μg of Lsa24 for 1 h 30 min prior to the addition of 1×10^7 leptospires. Wells were probed with anti-LipL32 serum. Data are expressed as the mean $A_{492} \pm$ the standard error of three independent experiments, each performed in triplicate. Significance was assessed by comparison with the no-protein wells by Student's two-tailed *t* test (∗, $P < 0.05$).

could be readily detected in the TX-114 detergent phase, indicating that this protein is a component of the leptospiral membrane (Fig. 8).

DISCUSSION

It is well known that pathogen adhesion to and colonization of host tissue is an early and critical event in the infection process. Although a number of putative spirochete virulence factors involved in toxin production, attachment, and immunity have been suggested, the role of the majority of those factors in pathogenesis remains uncertain. To date, a single leptospiral adhesin displaying fibronectin-binding properties has been described (26). Of unknown identity, this protein is specifically expressed on the surface of virulent *L. interrogans* serovar Icterohaemorrhagiae. In this work, we report for the first time an *L. interrogans* protein, named Lsa24, that binds strongly to laminin, a constituent of the basement membrane underlying both the epithelium and the endothelium. Lsa24 was expressed in *E. coli* as a 24-kDa full-length recombinant protein. The purified protein exhibited a single major band in SDS-PAGE suitable for ECM-binding assays. The structural integrity of the purified protein was assessed by CD spectroscopy, which revealed a predominant molecule population of β-strands. The presence of Lsa24 in the hydrophobic, detergent phase of *L. interrogans* Triton X-114-solubilized extract points out its localization at the bacterial membrane, most likely at the outer membrane, as previously shown for LipL32 (17, 18).

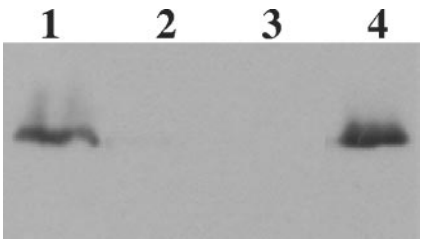

FIG. 8. Cellular localization of Lsa24. Triton X-114 fractions of *L. interrogans* serovar Copenhageni organisms were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with Lsa24 antiserum. Fractions analyzed were the whole organism (lane 1), Triton X-114-insoluble pellet (lane 2), aqueous phase (lane 3), and detergent phase (lane 4).

Laminin-1, classically referred as laminin, is a large, flexible protein composed of three very long polypeptide chains (α, β, and γ) arranged in the shape of an asymmetric cross and held together by disulfide bonds. A characteristic feature of laminin is its high carbohydrate content (13 to 15%) (13). Laminin recognition may play a role in the pathogenicity of microorganisms: virulence and adherence of *Trichomonas vaginalis* and *Trichomonas fetus* to epithelial cells is greatly increased in the presence of laminin (38). Based on an in vivo model for the study of fungal pathogenicity, Vicentini and colleagues demonstrated that the binding of *Paracoccidioides brasiliensis* to laminin enhanced the fungal pathogenesis (42).

Helicobacter pylori, particularly hemagglutinating strains, has been shown to bind specifically to this ECM component (41). Initial recognition and binding of laminin occur through surface-exposed lipopolysaccharide (40). Subsequently, a more specific interaction with a 25-kDa outer membrane adhesin on the bacterial surface occurs (41). To rule out the presence of residual *E. coli* LPS that could eventually interfere with our ECM-binding assays, all recombinant proteins used in our studies were treated with polymyxin B. A dose-dependent specific and saturable binding of Lsa24 to immobilized laminin was observed, fulfilling the properties of a typical receptor-ligand interaction. Metaperiodate oxidation of laminin caused significant reduction in the binding activity, strongly suggesting the involvement of laminin carbohydrate moieties in the interaction. This finding is in agreement with the previously published data pointing to a crucial role of laminin carbohydrate groups in the interaction with pathogens (5, 8, 10, 15, 35, 39, 41).

To date, no universal laminin-binding motif has been described within the known adhesins. Laminin-binding proteins do not share amino acid sequence similarity and vary in mo-

lecular size (5). Lsa24 seems to have no significant similarity with sequences currently available, including those from other spirochetes. In a recent report, Cameron and colleagues, using synthetic peptides, identified 10 amino acid residues present in a *T. pallidum* laminin-binding protein that are essential for attachment to laminin: amino acids $P^{98}V^{99}Q^{100}T^{101}$, $W^{127}I^{128}$, and $T^{182}A^{183}I^{184}S^{185}$ (7). Alignment of the Lsa24 amino acid sequence to this *T. pallidum* protein revealed no similarity (data not shown). Since no consensus laminin-binding motif has been described to date, it is possible that laminin-interacting sites harbored by Lsa24 might be specific and still need to be defined. Of all the ECM components tested, adhesiveness to laminin suggests that this highly glycosylated protein is the major ECM target for Lsa24.

In a previous report, Ito and Yanagawa demonstrated that virulent strains of *L. interrogans* attached to the ECM of mouse fibroblast cells (19). Moreover, it was suggested that leptospiral attachment to ECM correlates with virulence, since virulent lines of *L. interrogans* attached to ECM more effectively than intermediate virulent and avirulent lines of the same strains (19). From these results one may anticipate that *Leptospira* possesses proteins capable of adhering to several ECM components. We have demonstrated for the first time that this is indeed the case, as *L. interrogans* showed attachment to laminin, collagen type I, collagen type IV, cellular fibronectin, and plasma fibronectin.

As expected, Lsa24 partially inhibited attachment of intact *L. interrogans* to immobilized laminin. The inhibitory effect exerted by the recombinant protein was moderate and can be justified by the existence of additional *L. interrogans*-binding proteins contributing to the leptospiral adherence to laminin.

In conclusion, we have identified a leptospiral protein in the *L. interrogans* serovar Copenhageni genome sequence that exhibits attachment to laminin. Considering that leptospires are highly invasive microorganisms, there might be several other adhesins involved in the initial steps leading to infection. Studies concerning the underlying molecular mechanisms involved in adhesion are currently under way. Uncovering bacterium-host interactions at a molecular level not only will assist our understanding of the host physiology but also will facilitate the search for vaccine targets against leptospirosis.

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