A novel approach to identification of host ligand-binding proteins:

leptospiral outer-membrane protein microarray

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Leptospirosis is a zoonosis with worldwide distribution caused by pathogenic spirochetes belonging to the genus *Leptospira*. The leptospiral life cycle involves transmission via fresh water and colonization of the renal tubules of their reservoir hosts. Infection requires adherence to cell surfaces and extracellular matrix components of host tissues. These host-pathogen interactions involve outer membrane proteins (OMPs) expressed on the bacterial surface. In this study, we developed an *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 outer membrane protein microarray containing all predicted lipoproteins and transmembrane OMPs. A total of 401 leptospiral genes or their fragments were transcribed and translated *in vitro* and printed on nitrocellulose-coated glass slides. We investigated the potential of this protein microarray to screen for interactions between leptospiral OMPs and fibronectin (Fn). This approach resulted in identification of the recently described fibronectin-binding protein, LIC10258 (MFn8, Lsa66), and 14 novel fibronectin-binding proteins, denoted Microarray Fn-binding proteins (MFn). We confirmed fibronectin-binding of purified recombinant LIC11612 (MFn1), LIC10714 (MFn2), LIC11051 (MFn6), LIC11436 (MFn7), LIC10258 (MFn8, Lsa66), and LIC10537 (MFn9) by Far Western blot assays. Moreover, we obtained specific antibodies to MFn1, MFn7, MFn8 (Lsa66), and MFn9 and demonstrated that MFn1, MFn7, and MFn9 are expressed and surface-exposed under *in vitro* growth conditions. Further, we demonstrated that MFn1, MFn4 (LIC12631, Sph2), and MFn7 enable leptospires to bind fibronectin when expressed in the saprophyte, *L. biflexa*. Protein microarrays are valuable tools for high-throughput identification of novel host-ligand binding interactions.
proteins that have the potential to play key roles in virulence mechanisms of pathogens.
INTRODUCTION

Pathogenic *Leptospira* spp. have world-wide distribution and cause a zoonosis that is transmitted from reservoir hosts (typically rodents) to humans via water or contaminated soil. Leptospirosis is common in tropical and subtropical regions of the world and significantly impacts public health (11, 34, 53, 63). Leptospirosis also has significant adverse effects on the agricultural industry by causing abortions, infertility, and death in livestock (2, 29). Exposure of mucous membranes or damaged skin to water or soil contaminated with leptospires shed in animal urine can lead to a potentially fatal infection, characterized by jaundice, renal failure, and/or pulmonary hemorrhage affecting 350,000–500,000 humans annually (11, 40, 63, 96).

Host-pathogen interactions are generally mediated by surface-exposed outer membrane proteins (OMPs). The two major types of bacterial OMPs, outer membrane lipoproteins and transmembrane OMPs, differ in their structure and OM-integration strategies. Lipoproteins become associated with membranes in part via a hydrophobic interaction between the N-terminal lipid moieties (three fatty acids) and the phospholipids of the lipid bilayer (23, 38). In contrast, transmembrane OMPs are typically integrated into the lipid bilayer by amphipathic β-sheets arranged in a barrel-like structure (50, 88) with surface-exposed external loops contributing to host-ligand binding in some cases (21, 81, 84). The availability of the *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genome sequence (14, 72, 86) has facilitated *in silico* analysis methods to identify candidate OMPs, including lipoproteins (89) and transmembrane OMPs (7, 37).

The lifecycle of pathogenic leptospires involves interactions with various host tissues at multiple stages of infection, including: (i) adherence to host tissues, (ii)
penetration of host barriers, and (iii) evasion of the host defense (69, 77, 82).

Identification and characterization of novel proteins that mediate these stage-specific interactions is crucial to a molecular understanding of leptospiral pathogenesis. Leptospires bind to a variety of host ligands, including fibronectin, fibrinogen, collagen, laminin, and elastin, indicating that extracellular matrix (ECM)-binding OMPs, or adhesins, are likely to be expressed by these spirochetes (18, 19, 43, 46, 56). It is likely that leptospires express distinct adhesins during different stages of infection, such as the initial attachment, dissemination, and colonization stages.

Numerous leptospiral proteins, including LigA/B, Lsa21, Lsa27, Lsa63, 36-kDa fibronectin-binding protein, Lsa24 (LfhA=LenA), LenB-F, LipL32, Lp95, TlyC, OmpL37, Lp95, LipL53, Lsa20, Lsa66, Lsa33 and Lsa25 have been shown to bind host ligands in vitro (1, 4, 5, 8, 16, 19, 27, 41, 43, 55-57, 65, 67, 75, 76, 79, 92, 97, 98). It is apparent that a certain level of functional redundancy exists among leptospiral ECM-binding proteins, and it remains unclear to what extent each of these are required for interactions of leptospires with ECM proteins. Only the following proteins or their corresponding antibodies have been tested for their capacity to interfere with leptospiral adherence to ECM: Lsa24, LigA/B, Lsa63, OmpL37 and Lsa66 (8, 19, 56, 75, 79, 98). Only partial inhibition was observed for Lsa24, LigA/B, Lsa63, and Lsa66 (8, 19, 75, 98), which partially could be due to non-optimal conformation of the recombinant protein or low antibody titre. Nevertheless, these studies suggest not only that additional fibronectin, laminin, collagen, and elastin-binding proteins likely exist in *L. interrogans* but also that functional redundancy may be part of its survival and/or virulence mechanisms. A tool for high throughput screening for protein-host ligand interactions would greatly accelerate research on leptospiral pathogenicity mechanisms. Employing protein microarrays to identify
ligand-binding proteins is an innovative approach (51, 60, 73, 106) that could serve as a useful tool for elucidating host-pathogen interactions. In the microbiology field, proteome microarrays have mostly been used for serological studies to identify targets of the human or animal immune response during course of infection with the goal of discovering diagnostic antigens (6, 9, 15, 22, 25, 26, 42, 44, 54, 59, 68, 91, 94, 99, 101, 107). To date, a few reports have described protein microarrays as a tool to screen for proteins with host ligand binding capacities, both studies focusing on \textit{Streptococcus} (32, 61).

In this study, we present the results of high-throughput identification of candidate host-ligand binding proteins using a leptospiral OMP microarray containing 401 leptospiral proteins. Fifteen leptospiral proteins with fibronectin (Fn)-binding capacities were identified and denoted MFn-proteins for: \textit{Microarray Fn}-binding proteins. Only LIC10258 (MFn8) has previously been described as a fibronectin protein, Lsa66 (75). Fibronectin-binding capacities were confirmed by ligand-binding assays for all recombinant MFn-proteins analyzed: LIC10258 (MFn8), LIC10714 (MFn2), LIC11051 (MFn6), LIC11436 (MFn7), LIC10258 (MFn8, Lsa66), and LIC10537 (MFn9) proteins. Specific antisera for MFn1, MFn7, and MFn9 were obtained, which allowed us to demonstrate that MFn1, MFn7, and MFn9 are localized on the surface of \textit{in vitro} grown leptospires by surface proteolysis. Finally, we demonstrated that \textit{L. biflexa} transformed with MFn1, LIC12631 (MFn4, Sph2), and MFn7 gains the ability to acquire fibronectin on its surface in liquid culture. Herein, we present an effective approach for high-throughput identification and characterization of novel host-ligand binding proteins. It is anticipated that the OMP microarray approach will prove to be an effective tool for screening against various
host-ligands to identify novel OMPs with the potential to serve as adhesins, new serodiagnostic antigens and vaccine candidates.
Bacterial strains and growth conditions. *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was isolated from a patient during a leptospirosis outbreak in Salvador, Brazil (49) and utilized within six *in vitro* passages. *L. biflexa* serovar Patoc strain Patoc 1 (Paris strain) (78) was kindly provided by Mathieu Picardeau (Institut Pasteur, Paris, France). Leptospires were cultivated at 30°C in Probumin™ Vaccine Grade Solution (84-066-5, Millipore, Billerica, MA) diluted five-fold into autoclaved distilled water (80). The same solution was utilized to obtain Probumin-Agar plates. Competent *E. coli* NEB 5-α (New England Biolabs, Ipswich, MA), and BLR(DE3)pLysS (Novagen, Madison, WI) were used for cloning and expression, respectively. *E. coli* were grown in Luria-Bertani (LB) broth or on agar plates with 50 μg/ml carbenicillin, 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, 40 μg/ml kanamycin or 40 μg/ml spectinomycin (Sigma-Aldrich, St. Louis, MO) when appropriate.

*In silico* identification of *L. interrogans* outer membrane proteins. The flow chart shown in Fig. 1 summarizes the criteria and algorithms that were used to identify candidate lipoproteins and transmembrane OMPs in *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (33, 72). Ninety seven OMPs and thirteen proteins with leucine-rich repeats were included based on *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genome annotation (33, 72). The SpLip algorithm (89) was utilized to identify lipoproteins. OMPs are thought to lack long hydrophobic stretches because they would cause the protein to be retained in the inner membrane, thus preventing it from reaching the outer membrane (50). Therefore, proteins with more than three alpha-helical transmembrane domains were detected and eliminated.
using the TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Online versions of the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) (74) and LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) (47) programs were used to predict signal peptides. Transmembrane OMPs were selected using two β-barrel prediction programs, PRED-TMBB (http://biophysics.biol.uoa.gr/PRED-TMBB/) (7) and TMBETA-NET (http://psfs.cbrc.jp/tmbeta-net/) (37). Three hundred and sixty-six genes were included in the leptospiral OMP microarray based on the following criteria: (i) all predicted lipoproteins, (ii) presence of a signal peptide with signal peptidase (SPI or SPII) cleavage site, (iii) absence of more than three inner membrane-spanning α-helices, and (iv) prediction of at least six membrane-spanning β-strands by either PRED-TMBB or TMBETA-NET (7, 37).

Preparing leptospiral OMP microarrays. The leptospiral outer-membrane protein microarray was prepared at the Protein Microarray Laboratory, University of California, Irvine. Proteins included in the microarray are listed in Supplemental Table S1. Gene-specific primers were designed with a 20bp vector recombination site overlap and 20bp of gene specific sequences. The following fragments of leptospiral immunoglobulin-like (Lig) were included: LigB: domains 1-6; LigA: domains 7-13; LigB: carboxy-terminal domain (19); and LigB: domains 8-12 (20). Large genes (>3,000 bp; FnbpA, Lic11458, Lic1_SPN3200, Lic10497, Lic11028, Lic11739, Lic11990, Lic12901, Lic10125, Lic10464, Lic10465, Lic11755, Lic12048, Lic12259, Lic13101) were amplified in smaller segments (2 kb, denoted successively as -s1, -s2, -s3 and -s4, Supplemental Fig. S1) with a 150-bp overlap in each segment. In total, 401 leptospiral ORFs and their fragments were cloned into the pXT7 expression vector using a high-throughput cloning method as described previously (26). For assessing expression, the pXT7 vector incorporated a 5’ polyhistidine (His) epitope
and a 3' hemagglutinin (HA) epitope on each protein. One hundred to 200 ng of each purified plasmid was added to the Expressway cell-free expression system (Life Technologies) and proteins were expressed overnight at room temperature with shaking at 250 rpm. Tween-20 was added to the entire mixture to a final concentration of 0.05% and 1-2 nanoliters per spot were printed onto Onycyte nitrocellulose slides (Grace BioLabs, Bend, OR) using a Gene Machines Omni Grid 100 microarray printer (DigiLab, Inc., Holliston, MA). The diameter of each spot was 200 microns.

The genes or their fragments of following well-characterized host-ligand binding proteins were cloned and expressed as described above and included in the leptospiral OMP microarray: *Staphylococcus aureus* FnbpA and the FnbpA-D (743-862) binding region (102) as positive controls for fibronectin binding; *Staphylococcus epidermidis* SdrG and SdrG (273-597) fibrinogen-binding region (83), *S. aureus* ClfA (221-559) fibrinogen-binding region (39), and *S. aureus* CNA (30-344) collagen-binding region (103) as negative controls for fibronectin binding. A “no DNA” negative control with an empty plasmid vector provided the baseline signal for fluorescence readout. The following controls were printed by default by the service provider, which specializes in human serology approaches (UC Irvine): (1) serially diluted human IgG as a positive control to confirm reactivity of secondary antibodies and account for potentially nonviable hybridization steps (secondary and tertiary antibody binding, washing, etc.); and (2) serially diluted Epstein-Barr Virus nuclear antigen-1 (EBNA1) as a methodological control given the high prevalence of latent Epstein-Barr Virus infection in human populations. Quality of protein expression and spotting was assessed by probing for the N-terminal His-tag and C-terminal human influenza hemagglutinin, HA-tag. Microarrays were stored in a dessicator and used within 3 months after printing.
Probing and analysis of microarrays. To probe protein microarrays with fibronectin, slides were assembled onto a Proplate multi-array 8-well module with spring clips (Grace Biolabs) and re-hydrated with Odyssey blocking buffer (LI-COR, Lincoln, NE) overnight at 4°C. Wells were rinsed with phosphate buffered saline (PBS), pH 7.2 and 10 μg/ml of human plasma fibronectin (Sigma-Aldrich) in Odyssey blocking buffer or blocking buffer alone (as a control for specificity of ligand-binding and absence of non-specific signal from detection with antibodies) was added to the wells. Following 1.5 h incubation at room temperature with gentle shaking, wells were washed seven times with PBS+0.1% Tween 20 (PBS-T). Arrays were incubated for 1h at RT with rabbit polyclonal antibody recognizing human fibronectin (Sigma-Aldrich) diluted 1000-fold (determined empirically) in Odyssey blocking buffer followed by washing as described above. Antibody-binding was detected by incubating the arrays with Cy3-conjugated rabbit IgG (diluted 1:200 in Odyssey blocking buffer) for 1h at RT. Finally, wells were washed three-times with PBS-T, the slide-modules were then removed and slides washed seven-times with PBS-T, four times with distilled water, air-dried in the dark and scanned with a GenePix 4000A scanner (MDS, Sunnyvale California). Images were obtained using GenePix Pro, version 3.0 (MDS) software at high resolution (10-μm pixel size). Fluorescence intensities of each spot were calculated using ImageJ, version 1.44 software (http://rsb.info.nih.gov/ij/). ImageJ measures pixel intensities of each spot and converts them to numerical mean fluorescence intensities with values ranging from 0 to 256. The fluorescence of the background value surrounding each spot was subtracted. Fluorescence intensities from arrays that were probed with blocking buffer only were subtracted from arrays probed with human plasma fibronectin. Finally, fluorescence intensities of “no-DNA” negative control spots were subtracted from...
each protein spot. The mean fluorescence intensities (MFIs) from duplicate spots and three independent experiments were plotted on a chart and an arbitrary threshold of 10 MFIs was used to identify fibronectin-binding proteins.

Cloning, expression, and purification of recombinant Mfn1, Mfn2, Mfn6, Mfn7, Mfn8, and Mfn9. The genomic loci and the proposed names for the genes in parentheses are: Lic11612 (mfn1), Lic10714 (mfn2), Lic11051 (mfn6), Lic11436 (mfn7), Lic10258 (mfn8) and Lic10537 (mfn9). The genes encoding the eight predicted OMPs were cloned into the expression vector, pET-20b(+) (Novagen, San Diego, CA). All primer sequences for amplification from Fiocruz L1-130 DNA are listed in Table 1. PCR was performed with Phusion High Fidelity DNA Polymerase (Finnzymes, Woburn, MA) and the following conditions were used for amplification of mfn1: 98°C for 30 sec, 30 cycles at 98°C for 10 sec, 71°C for 30 sec, 72°C for 1 min 15 sec, followed by 72°C for 7 min and cooling to 4°C. PCR conditions to amplify mfn2, mfn8 and mfn9 were: 99°C for 2 min, 35 cycles at 98°C for 10 sec, 62°C for 30 sec, 72°C for 1 min 30 sec, followed by 72°C for 7 min and cooling to 4°C. PCR conditions to amplify mfn6 were: 98°C for 30 sec, 30 cycles at 98°C for 10 sec, 62°C for 30 sec, 72°C for 1 min 10 sec, followed by 72°C for 7 min and cooling to 4°C. PCR conditions to amplify mfn7 were: 98°C for 1 min, 30 cycles at 98°C for 10 sec, 61°C for 30 sec, 72°C for 1 min 25 sec, followed by 72°C for 7 min and cooling to 4°C. PCR products were purified by QIAquick PCR Purification kit (Qiagen, Valencia, CA) and digested with NdeI and XhoI or NdeI and SalI (New England Biolabs, Ipswich, MA) for mfn1, mfn2, mfn6, mfn7, mfn9 or mfn8, respectively and ligated to pET-20b(+) digested with either NdeI and XhoI or NdeI and SalI. The plasmids were used to transform E. coli NEB 5-α and purified using the QIAprep Spin Miniprep Kit (Qiagen). After confirming the presence of correct
inserts by restriction enzyme digestion, the plasmids were used to transform
competent *E. coli* BLR(DE3)pLysS. Cultures were grown to OD₆₀₀ ~0.5 and then
protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside.
The His-tagged recombinant proteins were purified under denaturing conditions and
MFn7 was also purified under native conditions with Ni-NTA Agarose (Qiagen)
according to the manufacturer’s instructions (QIAexpressionist manual).

**Gel electrophoresis, antibodies and blotting assays.** Protein samples were
boiled for 5 min in Novex NuPAGE sample buffer (Life Technologies, Carlsbad, CA)
in the presence of 2.5% β-mercaptoethanol and separated in Bis-Tris 4-12%
polyacrylamide gradient NuPAGE gels (Life Technologies). The polyclonal rabbit
sera specific for the following proteins are described elsewhere: FlaA2 (24), OmpL37
(80), and Sph2 (MFn4) (62). For production of polyclonal rabbit serum recognizing
MFn1, MFn7, MFn8, and MFn9, the respective purified recombinant proteins were
separated by preparative gel electrophoresis and excised from the gel. New Zealand
White rabbits were immunized (Pacific Immunology, Ramona, CA) with 0.25 mg of
gel-purified recombinant protein four times over a nine-week period, and serum was
collected one week after the final injection. To obtain antibodies recognizing MFn3
(Sph3), a GYWEEKRAELGKSK peptide from *L. interrogans* serovar Lai strain
56601 (LA4004, amino acid positions 98-113) was synthesized (Pacific Immunology)
and coupled to keyhole limpet hemocyanin carrier protein via an N-terminal cysteine
to immunize New Zealand white rabbits as described above. Rabbit polyclonal
antibody recognizing *L. biflexa* LPS was obtained from MyBiosource (San Diego,
CA).

For Western and Far-Western blotting, proteins were electro-transferred to a
polyvinylidenedifluoride (PVDF) Immobilon-P membrane (Millipore, Billerica, MA)
and blocked with 5% milk/PBS-T for 1 h at room temperature. For dot-blot, proteins were applied to 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA) using the Bio-Dot SF Microfiltration apparatus (Bio-Rad) and blocked in Pierce Protein-Free Blocking buffer, PFBb (Thermo Scientific, Rockford, IL) overnight at 4°C. For Far-Western and dot-blotting, membranes were incubated for 1 h at RT with 10 μg/ml of fibronectin in milk/PBS-T or PFBb, respectively. For all assays, membranes were probed with rabbit polyclonal antisera and bound antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) (GE Lifesciences, Buckinghamshire, England). The blots were visualized by enhanced chemiluminescence reagents according to the manufacturer’s instructions (Thermo Scientific).

**L. biflexa** Patoc I transformants. *L. interrogans* genes encoding candidate fibronectin-binding proteins were initially cloned downstream of the *L. interrogans flaB1* promoter in pRAT578. The *flaB1* promoter sequence in pRAT578 was amplified from *L. interrogans* Fiocruz L1-130 DNA with Phusion High Fidelity DNA Polymerase (Finnzymes, Woburn, MA) using the oligonucleotides *flaB1*p(Kp)-1F and *flaB1*p(Xh)-2R (Table 2). Genomic DNA was purified from *L. interrogans* with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The PCR conditions were an initial denaturation step of 98°C for 1 min, 30 cycles of 10 sec 98°C denaturation, 20 sec 62.2°C hybridization, and 20 sec 72°C extension steps, and a final 72°C extension for 1 min followed by cooling of the reaction to 4°C. The *flaB1* promoter amplicon was digested with *KpnI* and *XhoI* and inserted into the multicloning site of the plasmid pGKlep4 (35).

The *Lic11612* (*mfn1*), *Lic13198* (*mfn3*), *Lic12631* (*mfn4*), *Lic11436* (*mfn7*), *Lic10537* (*mfn9*) genes and the last 1998 nt of *Lic12952* (*mfn12*) gene were amplified
from genomic DNA of *L. interrogans* strain Fiocruz L1-130 using oligonucleotides described in Table 2. PCR was performed with Phusion High Fidelity DNA Polymerase (Finnzymes, Woburn, MA). The PCR conditions were as follows: 98°C for 1 min, 30 cycles at 98°C for 10 sec, 68°C for 30 sec, 72°C for 1 min 10 sec, followed by 72°C for 7 min and cooling to 4°C for *mfn1*; 98°C for 1 min, 30 cycles at 98°C for 10 sec, 66°C for 30 sec, 72°C for 1 min 30 sec, followed by 72°C for 7 min and cooling to 4°C for *mfn3* and *mfn4*; 98°C for 1 min, 30 cycles at 98°C for 10 sec, 63°C for 30 sec, 72°C for 1 min 10 sec, followed by 72°C for 7 min and cooling to 4°C for *mfn7*, *mfn9* and *mfn12*. Amplified genes were then purified, digested with *NdeI* and *XhoI* restriction enzymes, and inserted between the corresponding restriction sites of pRAT578. Recombinant plasmids were used to transform *E. coli* NEB 5-α and purified using the QIAprep Spin Miniprep Kit (Qiagen). After confirming the presence of the correct inserts by restriction enzyme digestion, the pRAT578/MFn1, pRAT578/MFn3, pRAT578/MFn4, pRAT578/MFn7, pRAT578/MFn9 and pRAT578/MFn12 plasmids were digested by *KpnI* and *XhoI* to release the DNA fragments containing *Prom flaB1* followed by inserted gene sequences. The *Prom flaB1mfn1*, *Prom flaB1mfn3*, *Prom flaB1mfn4*, *Prom flaB1mfn7*, *Prom flaB1mfn9*, *Prom flaB1mfn12* were then cloned into the *KpnI- XhoI* restriction sites of the *E. coli-L. biflexa* shuttle vector pRAT575. pRAT575 was derived by inserting the linkers pGGGTACC (*KpnI*) and pCCTCGAGG (*XhoI*) (New England Biolabs) into the *PvuII* and filled-in *NgoMIV* sites, respectively, of pSLe94 (10). Plasmid constructs were verified by DNA-sequencing (Laragen, Culver City, CA).

*E. coli-L. biflexa* was prepared for transformation as previously described (58). In brief, *L. biflexa* was grown at 30°C until the optical density reached 0.4-0.6 at 420 nm. Cells were centrifuged at 3000 x g at room temperature and washed by once in...
deionized water followed by centrifugation. After removing the supernatant, the bacteria were resuspended in deionized water to a final concentration of around $3 \times 10^{10}$ cells/ml. 100 μl of the suspended bacteria were added to 0.25 μg of plasmid DNA, and added to chilled electroporation cuvettes with a 0.2 cm gap. The cuvette was placed in the electroporation unit (Bio-Rad Gene Pulser II) and subjected to electroporation at a setting of 1.8 kV, 25 μF, and 200 Ω. After adding 1 ml of Probumin™ Vaccine Grade Solution, the bacteria were transferred to a 14 ml polypropylene tube and incubated for 24 hours at 30°C with shaking. The culture (0.3 ml) was plated onto Probumin-Agar plates containing 40 μg/ml of spectinomycin and incubated at 30°C until leptospiral colonies appeared (10-16 days). Colonies were inoculated into liquid Probumin™ Vaccine Grade Solution containing 40 μg/ml spectinomycin. The presence of correct inserts in L. biflexa transformants was verified by PCR using pRAT575F and pRAT575R primers (Table 2). PCR was performed with Taq DNA Polymerase (Qiagen). The PCR conditions were as follows: 98°C for 2 min, 35 cycles at 94°C for 30 sec, 51°C for 45 sec, 72°C for 2 min, followed by 72°C for 7 min and cooling to 4°C.

**ELISA of L. biflexa transformant binding to fibronectin.** Microtiter plates were coated with human plasma fibronectin and non-specific binding sites were blocked with PFBb. L. biflexa Patoc I transformant cultures were harvested by centrifugation at 2000 x g for 15 min at room temperature and resuspended in PBS-5mM MgCl$_2$ to a final concentration of $1 \times 10^9$ cells/ml and $1 \times 10^8$ cells were added to the microtiter wells. Plates were incubated at 30°C for 90 min, unbound leptospires were removed by four washes with PBS-5mM MgCl$_2$, and adherent cells were fixed with methanol at -20°C for 10 min. Fibronectin-bound leptospires were detected by probing with L. biflexa LPS monoclonal antibody (MyBioSource), developing with
HRP-conjugated anti-rabbit IgG (Novagen) and a tetramethylbenzidine substrate (Thermo Scientific), and recording by spectrophotometry at 450 nm.

**Fibronectin acquisition by live Patoc 1 transformants.** *L. biflexa* cultures were grown to densities of $1 \times 10^8$ - $5 \times 10^8$ cells/ml and $30 \mu g/ml$, $10 \mu g/ml$ or $1 \mu g/ml$ (final concentrations) of human plasma fibronectin was added to the cells and incubated overnight at 30°C. Absence of bacterial aggregation was confirmed by dark-field microscopy and cells were then harvested by centrifugation at 6,000 x g for 5 min at room temperature and washed twice with PBS. Western blotting (described above) was used to assess binding of fibronectin by *L. biflexa* transformants.
RESULTS

Selection of *L. interrogans* proteins for the outer-membrane protein microarray. Figure 1 illustrates our strategy for selecting potential surface-exposed proteins. 171 candidate transmembrane OMPs was selected by combining several computer prediction tools with prior genome sequence annotations using criteria described in the Materials and Methods section and Figure 1. All predicted lipoproteins (177) were included because while the bioinformatic algorithm, SpLip, is suitable for predicting lipidation of spirochetal proteins, it does not address the cellular destination of lipoproteins (89). All annotated Leucine-rich repeat (LRR) proteins (13) were included due to their known potential for protein-protein interactions.

Identification of fibronectin-binding proteins. To identify fibronectin (Fn) binding proteins, the microarray containing the whole cell-free expression sample was probed with human plasma fibronectin. Positive spots were identified by comparing mean fluorescence intensities, MFIs (Fig. 2). Fifteen leptospiral proteins with MFIs above the arbitrary threshold of 10 were identified as candidate fibronectin-binding proteins designated Microarray Fn-binding proteins: MFn1-MFn15 (Figure 3). Fourteen of these proteins have not previously been described as fibronectin binding proteins. One protein, MFn8, was recently identified as a novel fibronectin-binding protein, Lsa66 (75). As expected, the positive control, *S. aureus* FnbpA-D had the highest MFI value (Fig. 2 and 3). The complete list of microarray proteins in descending order according to their MFIs is presented in Supplemental Table S1. The MFI threshold can be lowered to include additional fibronectin binding protein candidates. Thus, one of the best characterized leptospiral fibronectin-binding
proteins, LigB domains 8-12 (20) exhibited considerable fibronectin-binding yielding an MFI value of 6.7115 (Supplemental Table S1). However, lowering the MFI-value threshold would increase the number of false positives. Because our aim was to identify proteins with the greatest likelihood of being true fibronectin-binding proteins, we focused our studies on proteins with MFIs above threshold of 10. Other previously studied leptospiral proteins that exhibit fibronectin binding activity had the following MFI values were obtained: Lsa21 (LIC10368) - 3.13, Lsa24 (LenA, LIC12906) - 7.41, LenB (LIC10997) - 0.35, LenC (LIC13006) - 4.96, LenD (LIC12315) - 4.1, LenE (LIC13467) - 1.7, LenF (LIC13248) - 1.87, LipL32 (LIC11352) - 1.96, OmpL37 (LIC12263) - 0.27, Lp95 (LIC12690) - 0.63, LipL53 (LIC12099) - 6.04. An important distinction between our assay, which involves immobilized proteins, and the previously described fibronectin binding proteins is that most of those were analyzed using freely-soluble proteins (4, 5, 16, 41, 43, 55, 56, 75, 76, 79, 92, 97). Differences between experimental approaches and fibronectin source (plasma fibronectin versus cellular fibronectin) could be responsible for the low MFI values obtained for some leptospiral fibronectin-binding proteins. Also, the affinity of some previously characterized leptospiral proteins for fibronectin (4, 5, 16, 19, 41, 43, 55, 56, 75, 76, 79, 92, 97) is considerably lower than that of FnbpA-D (102), which may explain why most of those proteins did not meet the MFI-threshold of our assay.

Validation of microarray data by blotting assays using recombinant proteins. To validate the fibronectin-MFn protein interaction and eliminate false negatives, recombinant MFn1, MFn2, MFn6, MFn7, MFn8 and MFn9 proteins were subjected to Far-Western (Fig. 4) and dot-blot ligand binding (Fig. 5) assays. All analyzed recombinant proteins bound fibronectin by Far-Western blot assay in a dose-
dependent manner. However, MFn7 had to be purified under native conditions for binding to be observed, which mostly occurred with MFn7 degradation products (Fig. 4). Binding capacities of recombinant MFn proteins were quantified by performing ligand dot blots with different amounts of rMFn proteins (Fig. 5A). As expected, the positive control, rFnbpA-D exhibited the strongest binding, followed by rMFn2, rMFn8, rMFn1, rMFn6 and rMFn9 in order of decreasing binding (Fig. 5A). The density of each spot is shown in Figure 5B, omitting analysis of FnbpA-D to allow clearer representation of rMFn-protein data. The differences between Fn-binding by MFns in this assay (Figure 5) and OMP microarray assay (Figures 2 and 3) are likely due to the fact that purified and denatured proteins (with exception of MFn7) were used in dot-blot ligand binding assay. Surprisingly, rMFn7n showed the weakest binding for higher amounts of rMFn7n (1, 0.5 and 0.25 μg) compared to stronger binding for lower amounts of rMFn7n (0.125 and 0.0625 μg) and finally decreasing again for the lowest protein amount, 0.03125 μg (Figure 5A and B). This pattern of results was unlikely to be an artifact as it was reproduced in several independent experiments. One possible interpretation of these data is that higher protein concentrations interfere with rMFn7n fibronectin binding abilities by inhibiting proper folding of the protein. This interpretation suggests that the binding activity of MFn7 is conformationally dependent, requiring its native conformation for fibronectin interactions, while denatured protein does not bind the ligand (Fig 4). The negative control, BSA did not exhibit significant binding.

**MFn-proteins are localized on leptospiral surface.** Rabbit antisera recognizing MFn1, MFn7, MFn8 and MFn9 were obtained and utilized in Western blots to determine whether these proteins are expressed in cultivated *L. interrogans* Fiocruz L1-130 (Fig. 6). All sera recognized the corresponding recombinant proteins
according to their predicted molecular weight of 48 kDa for MFn1, 80 kDa for MFn7, 68 kDa for MFn8 and 75 kDa for MFn9 (Fig. 6). Immune rabbit sera recognized MFn1, MFn7, MFn9 proteins expressed by in vitro grown L. interrogans, with native MFn1 and MFn9 migrating as 45 kDa and 70 kDa bands, respectively (Fig. 6). Rabbit serum recognizing MFn8 gave ambiguous results as three times the usual number of L. interrogans cells had to be loaded into the gel to detect several weak bands (Fig. 6).

The pre-immune rabbit serum for each of these antigens was tested and did not recognize any bands (data not shown). The presence of several cross-reactive bands indicates that MFn8 is either not expressed or expressed in very low levels in cultivated L. interrogans Fiocruz L1-130. No detectable alteration in expression levels of MFn1, MFn7, MFn8 and MFn9 was observed in L. interrogans cultures treated with 120 mM NaCl (data not shown), which has previously been shown to induce expression of LigA, LigB, and Sph2 (62) by simulating physiological conditions found in the mammalian host. We selected MFn1, MFn7 and MFn9 antisera for surface localization studies by surface proteolysis using proteinase K treatment and found that all three proteins were cleaved by proteinase K in a dose-dependent manner (Fig. 7). OmpL37 was included as a positive control for surface-proteolysis (80) and was cleaved by the enzyme in a dose-dependent manner (Fig. 7). FlaA2 was used as a negative control and did not exhibit evidence of proteolysis in any of the proteinase-K concentrations applied (Fig. 7), confirming the integrity of the leptospiral outer membrane. The results indicate that MFn1, MFn7 and MFn9 are localized on the surface of L. interrogans. MFn3= Sph3 and MFn4= Sph2 were not subjected to surface localization analysis due to uncertainty about expression in L. interrogans (Fig. 8 D and E) or known release of Sph2 to the growth medium (62).
Other MFn proteins were not subjected to surface proteolysis due to lack of specific antibodies.

**L. biflexa/MFn-protein transformants.** *L. biflexa* transformants carrying *mfn1, mfn3, mfn4, mfn7, mfn9 or mfn12* were obtained. The presence of inserts of correct size in the pRAT575 vector was verified by PCR using pRAT575-specific oligonucleotides (Table 2) for all *L. biflexa* Patoc I transformants (data not shown). The expression of recombinant proteins by *L. biflexa* transformed with pRAT575 vector constructs containing the *mfn1, mfn3, mfn4, mfn7, mfn9 or mfn12* genes were not detected by Coomassie staining (data not shown). Therefore, transformants were subjected to further analysis by immunoblot (Fig. 8) utilizing rabbit serum raised against recombinant proteins or an *L. interrogans* peptide, as described in the Materials and Methods section. The lack of antibodies recognizing MFn12 prevented the assessment of MFn12 expression by *L. biflexa* transformants. Only overexpression of MFn1, MFn4 (Sph2) and MFn7 could be detected by their respective antibodies (Fig. 8). *L. biflexa* does not possess a homolog of MFn1, and a band corresponding to the predicted molecular weight (48 kDa) of MFn1 was present only in the *L. biflexa/MFn1* transformant (Fig. 8A, Lane Lb/1). Although *L. biflexa* does have a MFn7 homolog (LBF_1774, 32% identities), no expression was detected in control, *L. biflexa/pRAT575*, whereas overexpression of MFn7 was achieved in the *L. biflexa/MFn7* transformant (Fig. 8B, Lane Lb/7). Presumably, the homology between the *L. interrogans* MFn7 protein and its *L. biflexa* MFn7 homolog was not sufficient for antibody recognition. *L. biflexa* possesses a homolog of the MFn9 protein (LBF_2582, 52% identities), which was recognized with immune rabbit serum, and no overexpression was achieved by the *L. biflexa/MFn9* transformant (Fig. 8C, Lanes Lb/C and Lb/9).
Antisera recognizing two related leptospiral sphingomyelinases, MFn3 (Sph3) (this study) and MFn4 (Sph2) (62) were examined for their abilities to recognize either MFn3 or MFn4 in *L. biflexa* transformants (Fig. 8D and E). MFn4 expression was detected only by its homologous antiserum and neither antiserum detected MFn3 expression by *L. biflexa/MFn3* (Figure 8D and E). Although *L. biflexa* has neither MFn3 (Sph3) nor MFn4 (Sph2) homologs, several bands were recognized by MFn4 antiserum in both the control, *L. biflexa/pRAT575* as well as *L. biflexa/MFn3* and *L. biflexa/MFn4* (Fig. 8D). Of note, several bands observed in *L. biflexa* by MFn3 antiserum (Fig. 8E) were due to non-specific antibodies present in pre-immune serum of a rabbit that was used for immunization with the Sph3 peptide (data not shown). A 76 kDa band corresponding to predicted molecular weight of 71.7 kDa for Sph2 was recognized by Sph2 antiserum (Fig. 8D, lane Li). This band was not reported for *L. interrogans L1-130* grown under normal in vitro conditions (62). Identification of this new band could be due to differences in the media used for cultivation, as we used Probumin instead of EMJH medium. Notably, the 76 kDa band corresponds to the cell-associated low-intensity band described in *L. interrogans* serovar Pomona (17). The more prominent 63 kDa band that has been suggested to correspond to SphH (17, 62), was also detected in our study (Fig. 8D, Lane Li). Antiserum raised against the unique Sph3-peptide (Materials and Methods) recognized a unique 90 kDa band in *L. interrogans L1-130* whole cell lysates (Fig. 8E, Lane Li), which is considerably larger than the predicted MW of Sph3 (65 kDa). The lack of reports of Sph3 expression by *in vitro* cultivated leptospires makes it difficult to know whether this band corresponds to one of the sphingomyelinase-like proteins or is merely a cross-reactive band.
Validation of fibronectin-binding capacities by *L. biflexa* transformants expressing MFn-proteins. MFn-protein binding to fibronectin was assessed by testing the ability of *L. biflexa* transformants to acquire soluble fibronectin added to their growth medium. Immunoblot analysis revealed that *L. biflexa* transformants expressing MFn1, MFn4 and MFn7 bound substantially more soluble fibronectin from the culture media than control *L. biflexa* transformed with the pRAT575 empty vector (Fig. 9). As previously described, the *L. biflexa* wt strain binds low amounts of fibronectin (31), which is also apparent from our results (Fig. 9). However, densitometry analysis demonstrates that expression of MFn1, MFn4 and MFn7 dramatically increases binding of fibronectin by *L. biflexa* when supplied at concentrations ranging from 1 μg/ml to 30 μg/ml (Fig. 9). The greatest fold increase in fibronectin binding was achieved with the lowest fibronectin concentration of 1 μg/ml: *L. biflexa/MFn1* acquired 84.5 times more fibronectin, *L. biflexa/MFn4* acquired 16.53 times more fibronectin and *L. biflexa/MFn7* acquired 102.2 more fibronectin when compared to the control (Fig. 9A) after normalization using the intensity of the LPS band as a loading control (Fig. 9B). Acquisition of fibronectin supplied at 10 μg/ml or 30 μg/ml was increased to similar levels in *L. biflexa* expressing MFn1, MFn4 and MFn7, ranging from 3.0-fold to 4.8-fold (Fig. 9A). No substantial increase in fibronectin (10 μg/ml and 30 μg/ml) binding was observed for *L. biflexa* transformants expressing MFn3, MFn9 or MFn12 (Fig. 9A). While *L. biflexa* transformants expressing MFn3 and MFn12 increased binding of fibronectin (1 μg/ml) by 3.8 and 3.3-fold, respectively, this increase was not significant when compared to that of *L. biflexa* transformants expressing MFn1, MFn4 and MFn7 (Fig. 9).
The ability of *L. biflexa* transformants to bind fibronectin was also assessed by a whole-cell ELISA. However, no statistically significant enhancement in binding was observed by *L. biflexa* MFn protein transformants compared to *L. biflexa* controls transformed with pRAT575 (data not shown). This was most likely due to the fact that immobilized fibronectin was assayed as opposed to freely-soluble Fn utilized in other assays described herein.
Outer membrane proteins (OMPs) of diderm bacteria are of great interest because of their location on the cell surface where bacterial pathogens interact with the host. OMPs often play key roles in pathogenesis by acting as (i) adhesins, (ii) targets for bactericidal antibodies, (iii) receptors for various host molecules, and/or (iv) porins. In the case of pathogenic *Leptospira* species, OMPs are likely to be key mediators of these organisms’ adaptation to host tissues and their response to changes in environmental conditions during their life cycle. Leptospiral surface components are thought to recognize host molecules, counteract host defense mechanisms, and promote the invasion and colonization of various tissues. Therefore, the identification and characterization of OMPs is critical to the understanding of pathogenesis mechanisms, the development of diagnostic antigens, and the identification of potential vaccine candidates. The aim of this study was to develop a novel approach to high throughput identification of host-ligand binding proteins by designing a protein microarray consisting of potential surface-exposed proteins and screening the microarray for fibronectin binding proteins.

The OMP microarray approach proved to be a reliable method to screen for leptospiral fibronectin-binding proteins. *L. interrogans* proteins with the highest MFI values were confirmed as fibronectin-binding proteins by solid phase binding assays and by enhanced fibronectin acquisition when expressed in the saprophyte, *L. biflexa*, serving as a surrogate-host model system. The accuracy of the OMP microarray approach to screen for host ligand binding proteins was verified by the finding that the protein with the highest MFI value was the well-characterized fibronectin binding protein FnbpA from *S. aureus*. In these studies, we employed the FnbpA-D region
that exhibits the highest affinity towards fibronectin (102). Interestingly, of the fifteen leptospiral proteins exhibiting the highest level of fibronectin-binding after probing OMP microarray with human plasma fibronectin (Fig. 3), only one previously characterized fibronectin binding protein, Lsa66 (75) exceeded the selected binding threshold. The fact that the reported affinities of previously characterized fibronectin binding proteins of *Leptospira*, including LigA/B, Lsa21, Lsa24 (LfbA=LenA), LenB-F, LipL32, Lp95, TlyC, LipL53, OmpL37, LipL53, and Lsa66 (4, 5, 16, 19, 41, 43, 55, 56, 75, 76, 79, 92, 97), are considerably lower than that of FnbpA-D (102) is consistent with their failure to meet binding-threshold established in our screen. Of all the leptospiral fibronectin-binding proteins studied to date, LigB is the most well characterized protein with the highest reported affinity for fibronectin (19, 20, 55, 56), and LigB domains 8-12 (20) exhibited considerable fibronectin-binding in our assay (Supplemental Table S1). While the OMP microarray approach is a very valuable tool for screening a large set of proteins for their interactions with host ligands, such a solid-phase assay in which proteins are immobilized on the nitrocellulose coated glass slides may potentially interfere with the proper folding and function of some proteins. As an example, it has been shown previously that immobilized OmpL37 binds elastin less efficiently than that freely soluble OmpL37 (79).

MFn2 and MFn5 are annotated as TonB-dependant receptor and TolC-family proteins, respectively. While experimental evidence supporting this designation is lacking, the predicted beta-strand structure indicate that these proteins are likely integrated in the outer-membrane and the external loops may interact with host-ligands similarly as it has been shown for P66 of *B. burgdorferi* (21). Interestingly, two leptospiral sphingomyelinase-like proteins, Sph3 (MFn3) and Sph2 (MFn4) were identified as fibronectin-binding proteins by the microarray assay (Fig. 2 and 3).
Sphingomyelinases hydrolyse sphingomyelin, a constituent of animal cell membranes, into ceramide and phosphorylcholine. Most bacteria do not produce sphingomyelin, therefore it is thought that bacterial sphingomyelinases target the host membrane to promote infectivity as shown for *S. aureus* and *Listeria ivanovii* (13, 36). Leptospiral sphingomyelinase-like proteins Sph1, Sph4 and SphH lack essential enzymatic residues (70, 71), while cytotoxic effects have been described for Sph2 and SphH (52, 105). Until now, host-ligand binding activity had not been described for Sph2; however, non-cytotoxic biological activities of leptospiral sphingomyelinases were thought likely to exist and could play roles in pathogenesis mechanisms (71). In fact, leptospiral sphingomyelinase-like proteins have been proposed to be involved in binding host ligands (71). This is supported by the example of TlyC, the leptospiral hemolysin-like protein, which lacks haemolytic activity but exhibits binding to ECM components (16). A unique role of staphylococcal sphingomyelinase in biofilm formation has been reported (45), indicating that sphingomyelinases can have multiple functions. Since Sph2 is believed to be secreted (62), it is tempting to speculate that it may function similarly to the extracellular adhesion protein (Eap) of *S. aureus* (3).

Validation of results obtained with a novel screening method such as the protein microarray is essential. Therefore, we studied the fibronectin binding capacities of nine out of fifteen leptospiral proteins with MFI values above 10 that are summarized in Table 3. Fibronectin binding was confirmed for MFn1, MFn2, MFn6, MFn7, MFn8 and MFn9 protein by solid phase binding assays (Fig. 4 and 5). The biological significance of the MFn1, MFn7, MFn8 and MFn9 was assessed as only proteins exposed on the leptospiral surface would have the ability to interact with ECM components in the host. We were able to show that MFn1, MFn7 and MFn9 are
localized on the surface of pathogenic *Leptospira* (Fig. 7). In our study, MFn8 appears not to be expressed by cultivated *L. interrogans* (Fig. 6). It is important to note that a previous study on MFn8 (Lsa66) failed to provide convincing evidence for synthesis of the protein in cultivated *L. interrogans*. The study lacked an immunoblot with leptospiral cell lysates. Moreover, the liquid phase IFA data presumed to demonstrate surface exposure of Lsa66 showed a single leptospiral cell with one peripheral fluorescent dot that cannot be distinguished from a random fluorescent particle (75). Therefore, the available data not only lack evidence for Lsa66 surface localization but also do not convincingly demonstrate its expression by cultivated leptospires (75). However, MFn8 does appear to be expressed *in vivo* as leptospirosis patient sera recognize Lsa66 (75). Further, we demonstrated that *L. biflexa* MFn1, MFn4 and MFn7 transformants substantially increase acquisition of freely-soluble fibronectin by live cells (Fig. 9), supporting the role of these proteins in host-pathogen interactions.

Whole proteome or selected protein microarrays are increasingly used in infectious disease research to identify new biomarkers that are either involved in the disease process or that are targets of immune responses (6, 9, 12, 15, 22, 25, 26, 42, 44, 48, 51, 54, 59, 60, 68, 73, 85, 91, 94, 95, 99-101, 106, 107). Compared to other proteomic techniques such as two-dimensional gel electrophoresis and mass spectrometry, the arrayed proteins are selected from the genome sequence, facilitating high-throughput applications that require examination of the entire or partial proteome of an infectious agent. A cell-free coupled transcription/translation reaction has been previously reported as a rapid and successful method to develop protein microarrays against a number of infectious organisms (9, 26, 93), bypassing time- and labor-intensive purification steps. Our OMP microarray was obtained using a system where both transcription and translation occur in a single reaction chamber to synthesize proteins.
from cloned PCR products followed by printing the whole reaction directly on glass slides. This is a first report on utilizing such an approach to identify host-ligand binding proteins.

More conventional approaches for screening for proteins involved in protein-protein interactions include the phage display method (87, 90) and yeast two-hybrid (Y2H) system (30, 66). While these methods have been widely used (66), the weaknesses of these approaches necessitate development of alternative methods, such as the OMP protein microarray approach that we present here. Our protein microarray consists of proteins expressed in cell-free expression system, which eliminates many of the pitfalls of phage-display and Y2H assays, most importantly undesirable interactions between proteins of interest and bacteriophage or yeast components. The OMP microarray has the added advantage of being able to be stored for several months, making the approach more practical. Moreover, the small format and numerous replicates allows screening with a very large set of various host-ligands and optimization by testing different ligand or detection agent concentrations within very small volumes of costly host-components or antibody conjugates. Nevertheless, some limitations are inherent for all these screening systems because some weakly expressed proteins may be undetected and proteins are produced externally of their respective organism. For example, the absence of post-translational protein modifications may adversely affect identification of functional proteins (64). The lack of post-translational modification is a limitation to be considered when screening bacterial OMPs for their interactions with host ligands. Thus, it has been shown that glycosylation plays an important role in the functionality of many surface proteins of *Campylobacter jejuni* (104, 108). It is possible that post-translational modification may be necessary for functional activity of some leptospiral OMPs, including adhesins. In
fact, it has been proposed that methylation could regulate the switch between an active/inactive state of leptospiral virulence factors (28). It is important to note that the results obtained from any screening methods have to be interpreted with caution and that careful validation of positive hits is essential before conclusions can be reached.

In summary, we present a novel approach to identification of infectious disease ligand-binding proteins. Our protein microarray approach has been specifically designed to screen for host-ligand binding proteins known to reside in bacterial outer-membrane. Compared to a whole proteome microarray, selection of OMPs by in silico prediction dramatically decreases the cost and complexity of the protein array, and simultaneously reduces the risk of false-positive hits. However, if discovery of serodiagnostic antigens is the desired, whole proteome microarrays would be more beneficial as immunogenic antigens are often sub-cellular. Additionally, whole proteome microarrays may include host-binding proteins with unexpected structural properties that would otherwise be excluded from proteins selected using bioinformatic approaches. Our innovative OMP microarray approach allowed us to identify fourteen novel and one previously characterized fibronectin-binding protein. Nine of these proteins that we have designated as Microarray Fn-binding (MFn) proteins were subjected to various assays to validate their fibronectin-binding capacities and seven MFns were verified as leptospiral fibronectin binding proteins (Table 3). Further studies will be required to characterize the other MFn-proteins and determine their functional roles. We show that protein microarrays can be effectively used to identify novel bacterial surface proteins with the capacity to bind host ligands. Therefore, we believe that this novel approach will be a great tool for the scientific
community to study various pathogenic microorganisms and their interactions with the host.
ACKNOWLEDGEMENTS

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REFERENCES


TABLE 1

Primers for amplification of z genes for expression in *E. coli*.

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<th>Gene</th>
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<sup>a</sup>Restriction sites (CATATG for NdeI, CTCGAG for XhoI) are underlined
**TABLE 2**

Oligonucleotides for cloning of *z* genes in *L. biflexa*.

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<th>Oligonucleotide</th>
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<sup>a</sup>Restriction sites (GGTACC for KpnI, CATATG for NdeI, CTCGAG for XhoI) are underlined.

<sup>b</sup>Forward primer, Z7_11436F (Table 1) used for cloning in both, *E. coli* and in *L. biflexa*.
TABLE 3
Summary of OMP microarray identified fibronectin-binding proteins.

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<th>Expression in cultivate L.</th>
<th>Surface proteolysis</th>
<th>Solid phase Fn binding&lt;sup&gt;b&lt;/sup&gt; in L.</th>
<th>Over-expression in L. biflexa</th>
<th>Presence of homologs in L. biflexa transformants</th>
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<td>Yes</td>
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<td>MFn2</td>
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<td>n.d.</td>
<td>n.d.</td>
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<td>n.a., Synthetic peptide</td>
<td>Yes, 90 kDa</td>
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<td>MFn4</td>
<td>n.a., N-terminal fragment (27-190) (62)</td>
<td>Yes, 76 n.d.</td>
<td>n.d.</td>
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<td>No</td>
<td>Yes</td>
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<td>MFn6</td>
<td>D, n.a.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>MFn7</td>
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<td>Yes, 80 kDa</td>
<td>Yes</td>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Yes</td>
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<td>MFn8</td>
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<td>n.d.</td>
<td>2</td>
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<td>MFn9</td>
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<td>5</td>
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<td>MFn12</td>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>No</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> d or n: purified under denaturating or native conditions, respectively. n.a.: not applicable.
b n.d.: not determined

c ordered dose dependent strength of binding: 1=strongest

d Strongest binding at lower amounts, receptor-like characteristics; only native protein binds

54
Figure 1

Selection of OMP microarray candidates. Three hundred and sixty-one genes were included in the leptospiral OMP microarray. All annotated lipoproteins (177), OMPs (97) and Leucine-rich-repeat proteins (13) were included. Additional transmembrane OMPs were identified by the following criteria: (i) presence of a signal peptide with signal peptidase cleavage site by http://www.cbs.dtu.dk/services/SignalP and (ii) absence of more than three inner membrane-spanning α-helices by TMHMM (www.cbs.dtu.dk/services/TMHMM), and (iii) prediction of at least six membrane-spanning β-strands by either PRED-TMBB http://biophysics.biol.uoa.gr/PRED-TMBB/ or TMBETA-NET http://psfs.cbrc.jp/tmbeta-net/.

Figure 2

Summary of screening the OMP microarray with fibronectin. One to two nl of 408 in vitro transcription-translation reactions were printed on the leptospiral OMP microarray. The microarray was probed with 10 μg/ml of fibronectin. Proteins binding to fibronectin were detected by rabbit serum against human fibronectin and antibody-binding was visualized by Cy3-conjugated rabbit IgG. The intensities of each spot were calculated using ImageJ, version 1.44 software (http://rsb.info.nih.gov/ij/). The error bars represent the standard deviation from three independent experiments.
Figure 3

Leptospiral OMP microarray proteins with the best fibronectin-binding activity.

The leptospiral OMP microarray was probed with 10 μg/ml of fibronectin. The mean fluorescence intensities were calculated using ImageJ, version 1.44 software ([http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). The annotation and MFI values of 15 leptospiral proteins that exhibited significant binding based on arbitrary threshold of 10 MFIs are shown. LIC11755-s1 and LIC11028-s4 denote fragments of these proteins that were cloned as 2 kb segments due to large size of these ORFs (>3,000 bp).

Figure 4

Ligand Affinity Blot (Far-Western) of rMFn-proteins. Recombinant proteins were separated by gel electrophoresis, blotted onto PVDF membrane and probed with 10 μg/ml of human plasma fibronectin. Recombinant OmpL37 (80) and *S. aureus* FnbpA D repeats (19, 102) were included as positive controls, and BSA was included as a negative control. Letters “n” or “d” denote purification of recombinant proteins either under native or denaturating conditions, respectively, as described in the Materials and Methods section as well as in previous reports (19, 80). The quantity of protein per lane is indicated (2 μg and/or 0.2 μg). The positions of molecular mass standards (in kilodaltons) are indicated on the left.

Figure 5

Semi-quantitative Dot-Blot. Data are representative of four independent dot-blot experiments. (A) Recombinant proteins were transferred to 0.45 μm nitrocellulose membrane by microfiltration and probed with 10 μg/ml of human plasma fibronectin. Micrograms of protein per spot are indicated on the left. BSA was included as a
negative control and the *S. aureus* FnbpA D repeat protein (19, 102) was included as a positive control. Only FnbpA-D (19) and MFn7 (Materials and Methods) were purified under native conditions. Duplicate spots were included in each experiment.

(B) The intensities of each spot were analyzed by ImageJ software by subtracting the background and measuring the mean density of the pixels in each spot. The mean values of duplicate spots are displayed.

**Figure 6**

Expression of MFn-proteins in cultivated *L. interrogans*. Lanes contain 1 x 10⁸ leptospires or 0.5 μg of recombinant proteins separated by gel electrophoresis, blotted onto PVDF membrane and probed with the rabbit immune sera specified below each blot. rMFn1, rMFn7, rMFn8, and rMFn9 denote the corresponding recombinant proteins. Lane WC contains *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 whole cell lysate. Asterisk indicates a three-fold increase in the amount of whole cell lysate (3 x 10⁸) loaded into wells. The identities of individual proteins are indicated on the right and the positions of molecular mass standards (in kilodaltons) are indicated on the left.

**Figure 7**

Surface localization of MFn-proteins. Intact spirochetes were incubated with different concentrations of proteinase K. Equivalents of 1 x 10⁸ leptospires per lane were separated by gel electrophoresis, transferred to a PVDF membrane, and probed with polyclonal rabbit antisera against MFn1, MFn7, MFn9, OmpL37, and FlaA2. The identities of individual proteins are indicated on the right, and the positions of molecular mass standard (in kilodaltons) are indicated on the left.
Expression of MFn-proteins by *L. biflexa* transformants. Whole cell lysates of 1 x 10^8 leptospires per lane were separated by gel electrophoresis, blotted to PVDF membrane and probed with rabbit immune sera recognizing: (A) MFn1, (B) MFn7, (C) MFn9, (D) MFn4 (Sph2), and (E) MFn3 (Sph3). Lanes Li: *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. Lanes Lb/C: *L. biflexa* serovar Patoc strain Patoc I transformed with an empty pRAT575 vector, used as a control. Lanes Lb/1, Lb/3, Lb/4, Lb/7, Lb/9: *L. biflexa* Patoc I transformed with pRAT575 vector constructs containing *mfn1*, *mfn3*, *mfn4*, *mfn7* and *mfn9* genes, respectively. The positions of molecular mass standards (in kilodaltons) are indicated on the left.

Acquisition of fibronectin by *L. biflexa* transformants. *L. biflexa* transformants were tested for their ability to acquire human plasma fibronectin. After washing to remove unbound ligand, 1 x 10^8 leptospires per lane were separated by gel electrophoresis, blotted onto PVDF membranes and probed with rabbit immune sera recognizing human fibronectin (A) or *L. biflexa* LPS (B). The intensities of the fibronectin and LPS bands were analyzed by ImageJ software by obtaining the percentage of the size of each peak. Relative densities of fibronectin bands were standardized to control, Lb/C (*L. biflexa* transformed with an empty pRAT575 vector) separately for each Fn concentration and further normalized against densities of corresponding LPS bands as a loading control. The data are representative of three independent experiments, performed separately.
Figure 1

L. interrogans Cop. L1-130 genome annotation

3531 CDS

SpLip

~ 450 IM-spanning alpha-helices
< 4 alpha-helices

Signalp + LipoP: Signal-peptide

~ 321 SP

PRED-TMBB & TMBETA-NET: OM-spanning beta-strands

~ 74 > 6 beta-strands

177 lipoproteins

97 OMPs + 13 LRR proteins

184 Integral OMPs
Figure 2
<table>
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<tr>
<th>Proteins</th>
<th>Mean fluorescence intensities</th>
<th>Annotation</th>
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<td>FnbpA binding region</td>
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<td><em>S. aureus</em> fibronectin-binding protein</td>
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<td>TolC family protein (MFn5)</td>
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<td>Leucine-rich repeat protein (MFn6)</td>
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<td>LIC13066</td>
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<td>LIC11028-s4</td>
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**Figure 3**
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9