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Leptospiral Outer Membrane Protein Microarray, a Novel Approach to Identification of Host Ligand-Binding Proteins

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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 freshwater 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 *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 OMP 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 the identification of the recently described fibronectin-binding protein, LIC10258 (MFn8, Lsa66), and 14 novel Fn-binding proteins, denoted Microarray Fn-binding proteins (MFns). We confirmed Fn 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, *Leptospira biflexa*. Protein microarrays are valuable tools for high-throughput identification of novel host ligand-binding proteins that have the potential to play key roles in the virulence mechanisms of pathogens.

Pathogenic *Leptospira* spp. have worldwide 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 to 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 life cycle 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 the 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 is 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

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partially could be due to nonoptimal conformation of the recombinant protein or low antibody titer. 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. The utilization of 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 *Streptococcus* (32, 61).

We present here 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 are denoted as MFn proteins (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 of the recombinant MFn proteins analyzed: LIC11612 (MFn1), 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 *in vitro*-grown leptospires by surface proteolysis. Finally, we demonstrated that *L. biflexa* transformed with MFn1, LIC12631 (MFn4 and Sph2), and MFn7 gains the ability to acquire fibronectin on its surface in liquid culture. We present here 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.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. 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 (catalog no. 84-066-5; Millipore, Billerica, MA) diluted 5-fold into autoclaved distilled water (80). The same solution was utilized to obtain Probumin-agar plates. Competent *Escherichia 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 of carbenicillin/ml, 12.5 μg of tetracycline/ml, 34 μg of chloramphenicol/ml, 40 μg of kanamycin/ml, or 40 μg of spectinomycin/ml (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

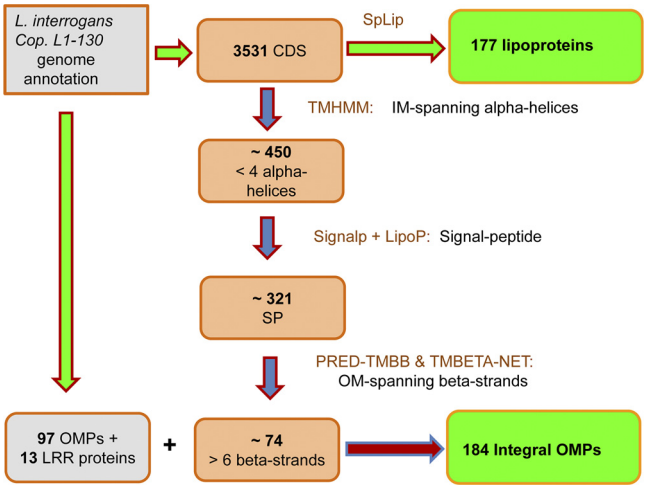

FIG 1 Selection of OMP microarray candidates. Three hundred and 61 genes were included in the leptospiral OMP microarray. All annotated lipoproteins ($n = 177$), OMPs ($n = 97$), and leucine-rich-repeat proteins ($n = 13$) were included. Additional transmembrane OMPs were identified by the following criteria: (i) the presence of a signal peptide with signal peptidase cleavage site by http://www.cbs.dtu.dk/services/SignalP and (http://www.cbs.dtu.dk/services/LipoP/, (ii) the absence of more than three inner membrane-spanning α-helices by TMHMM (www.cbs.dtu.dk/services/TMHMM), and (iii) the 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/).

in *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (33, 72). Ninety-seven OMPs and 13 proteins with leucine-rich repeats were included based on the *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 these 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). A total of 366 genes were included in the leptospiral OMP microarray based on the following criteria: (i) all predicted lipoproteins, (ii) the presence of a signal peptide with signal peptidase (SPI or SPII) cleavage site, (iii) the absence of more than three inner membrane-spanning α-helices, and (iv) the prediction of at least six membrane-spanning β-strands by either PRED-TMBB or TMBETA-NET (7, 37).

Preparing leptospiral OMP microarrays. The leptospiral OMP microarray was prepared at the Protein Microarray Laboratory, University of California, Irvine (UC-Irvine). The proteins included in the microarray are listed in Table S1 in the supplemental material. Gene-specific primers were designed with a 20-bp vector recombination site overlap and 20 bp of gene-specific sequences. The following fragments of leptospiral immunoglobulin-like (Lig) were included: LigB, domains 1 to 6; LigA, domains 7 to 13; LigB, carboxy-terminal domain (19); and LigB, domains 8 to 12 (20). Large genes (>3,000 bp; *FnbpA*, *Lic11458*, *Lic1_SPN3200*, *Lic10497*, *Lic11028*, *Lic11739*, *Lic11990*, *Lic12901*, *Lic10125*, *Lic10464*, *Lic10465*, *Lic11755*, *Lic12048*, *Lic12259*, and *Lic13101*) were amplified in smaller segments (2 kb, denoted successively as -s1, -s2, -s3, and -s4 [see Fig. S1 in the supplemental material]) 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. We added 100 to 200 ng of each purified plasmid 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 to 2 nl per spot were printed onto Oncyte 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 μm.

The genes or their fragments of the 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 FnbpA-D (positions 743 to 862) binding region (102) as positive controls for fibronectin binding and *Staphylococcus epidermidis* SdrG and SdrG (positions 273 to 597) fibrinogen-binding region (83), *S. aureus* ClfA (positions 221 to 559) fibrinogen-binding region (39), and *S. aureus* CNA (positions 30 to 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): (i) 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 (ii) 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. The quality of protein expression and spotting was assessed by probing for the N-terminal His tag and the C-terminal human influenza virus HA tag. Microarrays were stored in a desiccator and used within 3 months after printing.

Probing and analysis of microarrays. To probe protein microarrays with fibronectin, slides were assembled onto a Proplate multiarray 8-well module with spring clips (Grace Biolabs) and rehydrated 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 of human plasma fibronectin (Sigma-Aldrich)/ml in Odyssey blocking buffer or blocking buffer alone (as a control for specificity of ligand-binding and absence of nonspecific signal from detection with antibodies) was added to the wells. After 1.5 h of incubation at room temperature with gentle shaking, the wells were washed seven times with PBS plus 0.1% Tween 20 (PBS-T). The arrays were incubated for 1 h at room temperature with rabbit polyclonal antibody recognizing human fibronectin (Sigma-Aldrich) diluted 1,000-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 1 h at room temperature. Finally, the wells were washed three times with PBS-T, the slide modules were then removed, and the slides were 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, CA). Images were obtained using GenePix Pro (version 3.0; MDS) software at high resolution (10-μm pixel size). The fluorescence intensities of each spot were calculated by using ImageJ, version 1.44 software (http://rsb.info.nih.gov/ij/). ImageJ measures the pixel intensities of each spot and converts them to numerical mean fluorescence intensities (MFIs) with values ranging from 0 to 256. The fluorescence of the background value surrounding each spot was subtracted. The fluorescence intensities from arrays that were probed with blocking buffer only were subtracted from arrays probed with human plasma fibronectin. Finally, the fluorescence intensities of "no DNA" negative control spots were subtracted from each protein spot. The 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.

TABLE 1 Primers for amplification of genes for expression in *E. coli*

Oligonucleotide	Sequence (5′–3′)[a]	Gene
Z1_11612F	ATGTACATATGGAAGCGGGTGGACTTA	*mfn1*
Z1_11612R	GTAAACTCGAGTTCCAATTCCCAGGAT	*mfn1*
Z2_10714F	GAAGTTCATATGCAATCAGAAGAAACAAA	*mfn2*
Z2_10714R	AACAACTCGAGAAGTTCAAACGTGG	*mfn2*
Z6_11051F	GAAACATATGAAACACTACCTAACCT	*mfn6*
Z6_11051R	GTAGAACTCGAGAGAAAGATAAAGTACT	*mfn6*
Z7_11436F	AAGTTACATATGAAATACTTGACTGAAGA	*mfn7*
Z7_11436R	AAAGACTCGAGTTCCACATAAATTT	*mfn7*
Z8_10258F	CACTTCATATGAATACTTTTTTAAAGACG	*mfn8*
Z8_10258R	CATATGTCGACAAGTGAAAGATAAAAAT	*mfn8*
Z9_10537F	GTCTTCATATGGAGAATAAAAATTCTTCC	*mfn9*
Z9_10537R	AAAAAACTCGAGTTTACTTTTTTTCAAAATC	*mfn9*

[a] Restriction sites (CATATG for NdeI, CTCGAG for XhoI) are underlined.

Cloning, expression, and purification of recombinant Mfn1, Mfn2, Mfn6, Mfn7, Mfn8, and Mfn9. The genomic loci proposed names for the genes (in parentheses) were as follows: *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 of the primer sequences for the 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 the amplification of *mfn1*: 98°C for 30 s, 30 cycles of 98°C for 10 s, 71°C for 30 s, and 72°C for 1 min 15 s, followed by 72°C for 7 min and cooling to 4°C. The PCR conditions used to amplify *mfn2*, *mfn8*, and *mfn9* were as follows: 99°C for 2 min, 35 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 1 min 30 s, followed by 72°C for 7 min and cooling to 4°C. The PCR conditions used to amplify *mfn6* were as follows: 98°C for 30 s, 30 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 1 min 10 s, 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 s, 61°C for 30 s, and 72°C for 1 min 25 s, followed by 72°C for 7 min and cooling to 4°C. The PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and digested with NdeI and XhoI or NdeI and SalI (New England BioLabs) for *mfn1*, *mfn2*, *mfn6*, *mfn7*, *mfn9*, or *mfn8*, respectively, and ligated to pET-20b(+) digested with either NdeI/XhoI or NdeI/SalI. The plasmids were used to transform *E. coli* NEB 5-α and purified by using a QIAprep spin miniprep kit (Qiagen). After the presence of the correct inserts was confirmed by restriction enzyme digestion, the plasmids were used to transform competent *E. coli* BLR(DE3)pLysS. Cultures were grown to an optical density at 600 nm of ~0.5, and then protein expression was induced with 0.4 mM IPTG (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 to 12% polyacrylamide gradient NuPAGE gels (Life Technologies). The polyclonal rabbit sera specific for the following proteins are described elsewhere as indicated: FlaA2 (24), OmpL37 (80), and Sph2 (MFn4) (62). For the 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 9-week period, and serum was collected 1 week after the final injection. To obtain antibodies recognizing MFn3 (Sph3), peptide GYWEEEKRAELGKSK from *L. interrogans* serovar Lai strain 56601 (LA4004, amino acids 98 to 113) was synthesized (Pacific Immu-

TABLE 2 Oligonucleotides for cloning of genes in *L. biflexa*

Oligonucleotide	Sequence (5′–3′)[a]	Gene[b]
flaB1p(Kp)-1F	CTCGCTGGTACCGATCGAACCTAAGATTAGCTCA	$Prom_{flaB1}$
flaB1p(Xh)-2R	GTCCTCGAGTTCCATATGTTTCCTCCTTGAAACTGATC	$Prom_{flaB1}$
Z1_11612**f**F	ACGTCATATGTTTCAAAACGTTTTCAA	*mfn1*
Z1_11612**S**R	AGTACTCGAGTTATTCCAATTCCCAGG	*mfn1*
Z3_13198**f**F	ATGTCATATGAAAACAAAACGAAATC	*mfn3*
Z3_13198**S**R	GTAAAACTCGAGCTAACGATAAATTAGATC	*mfn3*
Z4_12631F	AGAGACATATGATAAACAAAATAACAAAACC	*mfn4*
Z4_12631**S**R	AGGACTCGAGTTAGCGATAAATAAGAT	*mfn4*
Z7_11436**S**R	ATAAACTCGAGTTATTCCACATAAATT	*mfn7*
Z9_10537**f**F	GAAAACATATGCATACCCTGCT	*mfn9*
Z9_10537**S**R	ATAAACTCGAGTTATTTACTTTTTTTCAAAA	*mfn9*
Z12_11028s4F	GCTCATATGTATCGCTCTTTTTTAA	*mfn12*
Z12_11028s4R	TTATCCTCGAGCTATTCTTCTAATATAATACT	*mfn12*
pRAT575_MPForv	CTAAATCGGAACCCTAAAGG	pRAT575 inserts
pRAT575_MPRev	CCACAATCAGACAATGACC	pRAT575 inserts

[a] Restriction sites (GGTACC for KpnI, CATATG for NdeI, and CTCGAG for XhoI) are underlined.
[b] The forward primer Z7_11436F (Table 1) was used for cloning in both *E. coli* and *L. biflexa*.

nology) 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* lipopolysaccharide (LPS) was obtained from MyBioSource (San Diego, CA).

For Western and far-Western blotting, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) and blocked with 5% milk–PBS-T for 1 h at room temperature. For dot blotting, proteins were applied to 0.45-μm-pore-size nitrocellulose membrane (Bio-Rad, Hercules, CA) using a 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 blot analyses, membranes were incubated for 1 h at room temperature with 10 μg of fibronectin/ml in milk–PBS-T or PFBb, respectively. For all assays, the membranes were probed with rabbit polyclonal antisera, and bound antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG; GE Life Sciences, 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) using the oligonucleotides flaB1p(Kp)-1F and flaB1p(Xh)-2R (Table 2). Genomic DNA was purified from *L. interrogans* with a Wizard genomic DNA purification kit (Promega, Madison, WI). The PCR conditions were as follows: an initial denaturation step of 98°C for 1 min, followed by 30 cycles of denaturation for 10 s at 98°C, hybridization for 20 s at 62.2°C, and extension for 20 s at 72°C, and a final 72°C extension for 7 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*), and *Lic10537* (*mfn9*) genes and the last 1,998 nucleotides of the *Lic12952* (*mfn12*) gene were amplified from genomic DNA of *L. interrogans* strain Fiocruz L1-130 by using the oligonucleotides listed in Table 2. PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes). The PCR conditions were as follows: 98°C for 1 min, 30 cycles at 98°C for 10 s, 68°C for 30 s, and 72°C for 1 min 10 s, 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 s, 66°C for 30 s, and 72°C for 1 min 30 s, followed by 72°C for 7 min and cooling to 4°C for *mfn3* and *mfn4*; and 98°C for 1 min, 30 cycles at 98°C for 10 s, 63°C for 30 s, and 72°C for 1 min 10 s, 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 the presence of the correct inserts was confirmed 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 $Promfla_{B1}$, followed by inserted gene sequences. $Promfla_{B1}$ *mfn1*, $Promfla_{B1}$ *mfn3*, $Promfla_{B1}$ *mfn4*, $Promfla_{B1}$ *mfn7*, $Promfla_{B1}$ *mfn9*, $Promfla_{B1}$ *mfn12* 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 pGGGTACCC (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).

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 to 0.6 at 420 nm. The cells were centrifuged at 3,000 × *g* at room temperature and washed by once in deionized water followed by centrifugation. After the supernatant was removed, the bacteria were resuspended in deionized water to a final concentration of around 3×10^{10} cells/ml. Then, 100 μl of the suspended bacteria was 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 1 ml of Probumin vaccine-grade solution was added, the bacteria were transferred to a 14-ml polypropylene tube and incubated for 24 h at 30°C with shaking. The culture (0.3 ml) was plated onto Probumin-agar plates containing 40 μg of spectinomycin/ml, followed by incubation at 30°C until leptospiral colonies appeared (10 to 16 days). The colonies were inoculated into liquid Probumin vaccine-grade solution containing 40 μg of spectinomycin/ml. The presence of the 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 s, 51°C for 45 s, and 72°C for 2 min, followed by 72°C for 7 min and cooling to 4°C.

Enzyme-linked immunosorbent assay (ELISA) of *L. biflexa* transformant binding to fibronectin. Microtiter plates were coated with human plasma fibronectin, and nonspecific binding sites were blocked with PFBb. *L. biflexa* Patoc I transformant cultures were harvested by centrifugation at 2,000 × *g* for 15 min at room temperature and resuspended in PBS–5 mM $MgCl_2$ to a final concentration of 10^9 cells/ml, and then 10^8

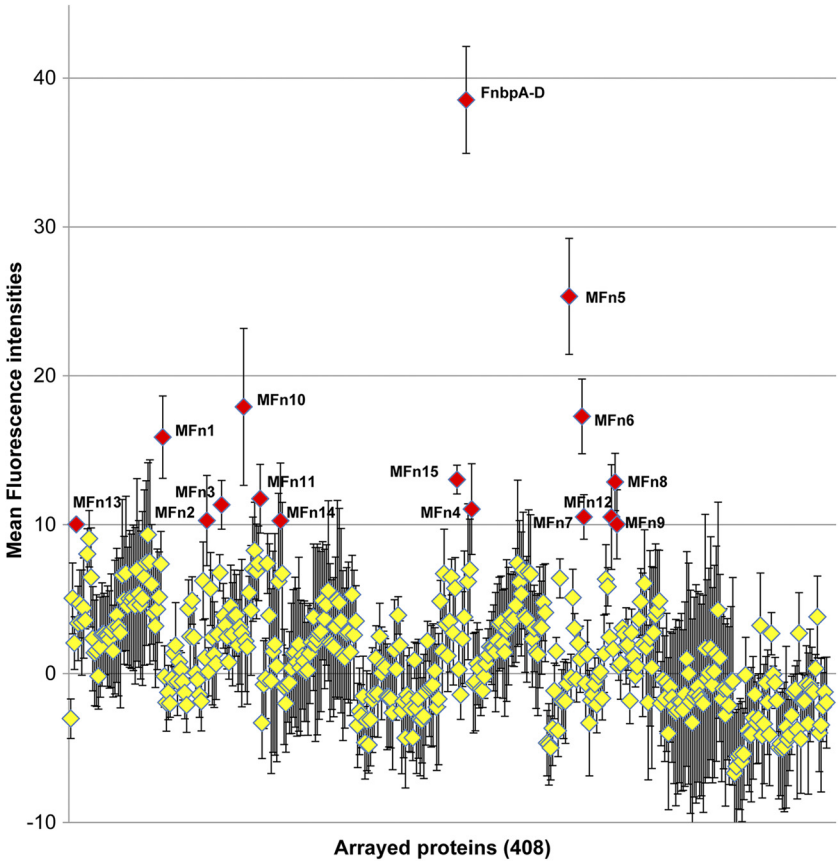

FIG 2 Summary of screening the OMP microarray with fibronectin. One to two nanoliters of 408 *in vitro* transcription-translation reactions were printed on the leptospiral OMP microarray. The microarray was probed with 10 μg of fibronectin/ml. Proteins binding to fibronectin were detected by using rabbit serum against human fibronectin, and antibody binding was visualized by using 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.

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–5 mM $MgCl_2$, and adherent cells were fixed with methanol at −20°C for 10 min. Fibronectin-bound leptospires were detected by probing the samples with *L. biflexa* LPS monoclonal antibody (MyBioSource), development with horseradish peroxidase-conjugated anti-rabbit IgG (Novagen) and a tetramethylbenzidine substrate (Thermo Scientific), and recording by spectrophotometry at 450 nm.

Fibronectin acquisition by live Patoc I transformants. *L. biflexa* cultures were grown to densities of 1×10^8 to 5×10^8 cells/ml, and then a 30-, 10-, or 1-μg/ml final concentration of human plasma fibronectin was added to the cells, followed by incubation overnight at 30°C. The absence of bacterial aggregation was confirmed by dark-field microscopy, and the cells were then harvested by centrifugation at 6,000 × *g* for 5 min at room temperature and washed twice with PBS. Western blotting (described above) was used to assess the binding of fibronectin by *L. biflexa* transformants.

RESULTS

Selection of *L. interrogans* proteins for the OMP microarray. Figure 1 illustrates our strategy for selecting potential surface-exposed proteins. A total of 171 candidate transmembrane OMPs were selected by combining several computer prediction tools with prior genome sequence annotations using criteria described in Materials and Methods and Fig. 1. All predicted lipoproteins ($n = 177$) were included because while the bioinformatic algorithm, SpLip, is suitable for predicting the 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 the MFIs (Fig. 2). Fifteen leptospiral proteins with MFIs above the arbitrary threshold of 10 were identified as candidate fibronectin-binding proteins designated MFn1 to MFn15 (Fig. 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, *Staphylococcus aureus* FnbpA-D had the highest MFI (Fig. 2 and 3). The complete list of microarray proteins in descending order according to their MFIs is presented in Table S1 in the supplemental material. 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 to 12 (20) exhibited considerable fibronectin-binding yielding an MFI of 6.7115 (see Table S1 in the supplemental material). However, lowering the MFI threshold

Proteins	Mean fluorescence intensities	Annotation
FnbpA binding region	38.53419	*S. aureus* fibronectin-binding protein
LIC13135	25.33956	TolC family protein (MFn5)
LIC11755-s1	17.91858	Hypothetical lipoprotein (MFn10)
LIC11051	17.2769	Leucine-rich repeat protein (MFn6)
LIC11612	15.88417	Hypothetical protein (MFn1)
LIC13066	13.03152	Hypothetical protein (MFn15)
LIC10258	12.87173	OmpA –related, Lsa66 (MFn8)
LIC11028-s4	11.74858	Tol transport system comp. (MFn11)
LIC13198	11.33758	Sphingomyelinase 3, Sph3 (MFn3)
LIC12631	11.04769	Sphingomyelinase 2, Sph2 (MFn4)
LIC11436	10.52173	LipL-45 related protein (MFn7)
LIC12952	10.51506	Hypothetical protein (MFn12)
LIC10714	10.27925	TonB-dependent receptor (MFn2)
LIC10713	10.26533	Iron regulated lipoprotein, LruB (MFn14)
LIC10537	10.0254	OmpA family protein (MFn9)
LIC11893	10.02483	CreD-like protein (MFn13)

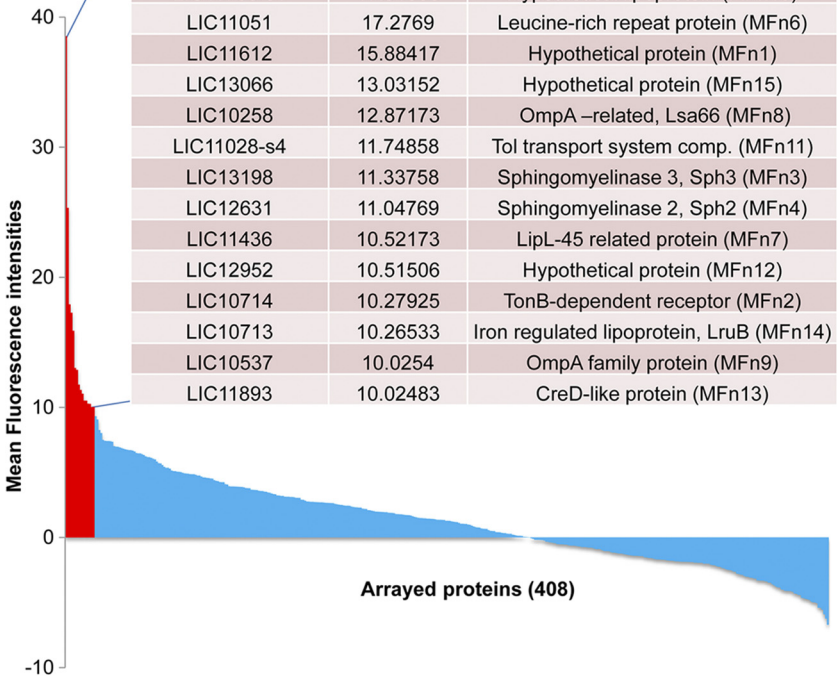

FIG 3 Leptospiral OMP microarray proteins with the best fibronectin-binding activity. The leptospiral OMP microarray was probed with 10 μg of fibronectin/ml. The MFIs were calculated using ImageJ, version 1.44 software (http://rsb.info.nih.gov/ij/). The annotation and MFI values of 15 leptospiral proteins that exhibited significant binding based on an 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 sizes of these ORFs (>3,000 bp).

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 a threshold of 10. Other previously studied leptospiral proteins that exhibit fibronectin binding activity with the following MFIs 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; and 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, 19, 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). The 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 Fig. 5B, omitting analysis of FnbpA-D to allow clearer representation of rMFn-protein data. The differences between Fn binding by MFns in this assay (Fig. 5) and the OMP microarray assay (Fig. 2 and 3) are likely due to the fact that purified and denatured proteins (with exception of MFn7) were used in a 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 (Fig. 5). This pattern of results was unlikely to be an artifact since 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 inhib-

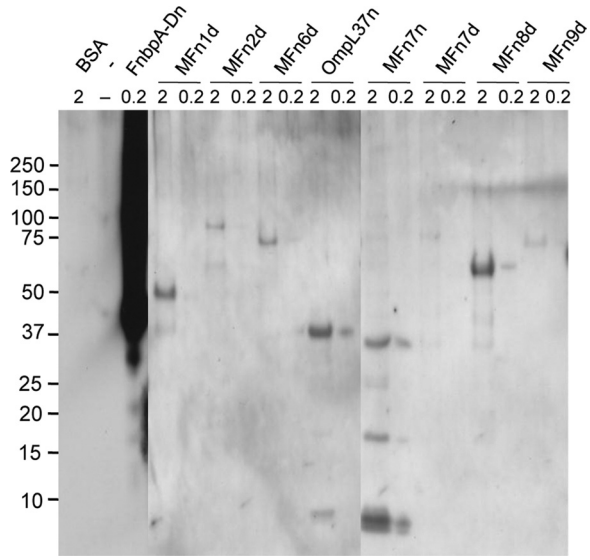

FIG 4 Ligand affinity blot (far-Western) of rMFn proteins. Recombinant proteins were separated by gel electrophoresis, blotted onto PVDF membranes, and probed with 10 μg of human plasma fibronectin/ml. Recombinant OmpL37 (80) and *S. aureus* FnbpA D repeats (19, 102) were included as positive controls, and BSA was included as a negative control. An "n" or a "d" denote purification of recombinant proteins either under native or denaturing conditions, respectively, as described in Materials and Methods, as well as in previous reports (19, 80). The quantity of protein per lane is indicated (2 or 0.2 μg). The positions of molecular mass standards (in kilodaltons) are indicated on the left.

iting proper folding of the protein. This interpretation suggests that the binding activity of MFn7 is conformationally dependent, requiring its native conformation for fibronectin interactions, whereas denatured protein does not bind the ligand (Fig. 4). The negative control, bovine serum albumin (BSA), did not exhibit significant binding.

MFn proteins are localized on the 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 masses 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, and MFn9 proteins expressed by *in vitro* grown *L. interrogans*, with native MFn1 and MFn9 migrating as 45- and 70-kDa bands, respectively (Fig. 6). Rabbit serum recognizing MFn8 gave ambiguous results since three times the usual number of *L. interrogans* cells had to be loaded into the gel to detect several weak bands (Fig. 6). The preimmune 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 the 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 the 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.

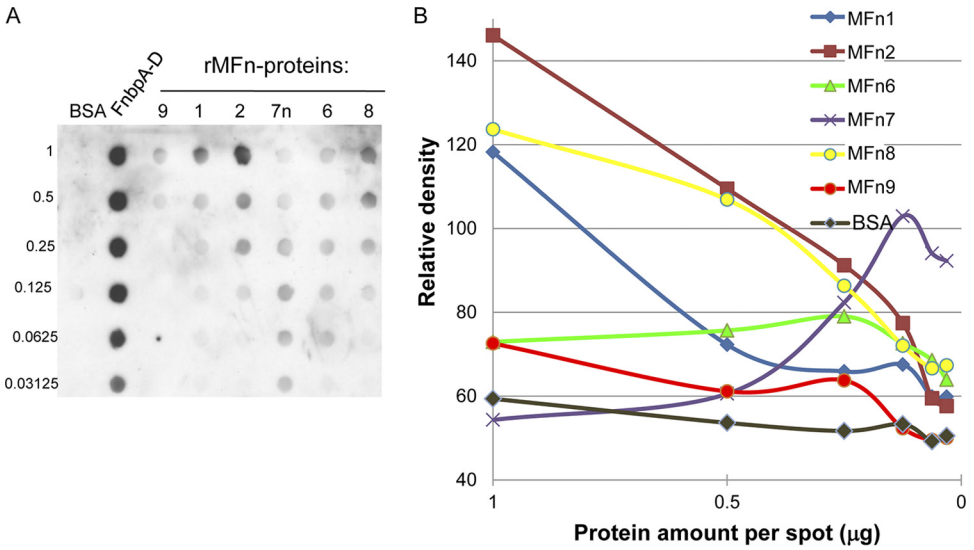

FIG 5 Semiquantitative dot blot. The data are representative of four independent dot blot experiments. (A) Recombinant proteins were transferred to a 0.45-μm-pore-size nitrocellulose membrane by microfiltration and probed with 10 μg of human plasma fibronectin/ml. The micrograms of protein per spot are 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 (see 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.

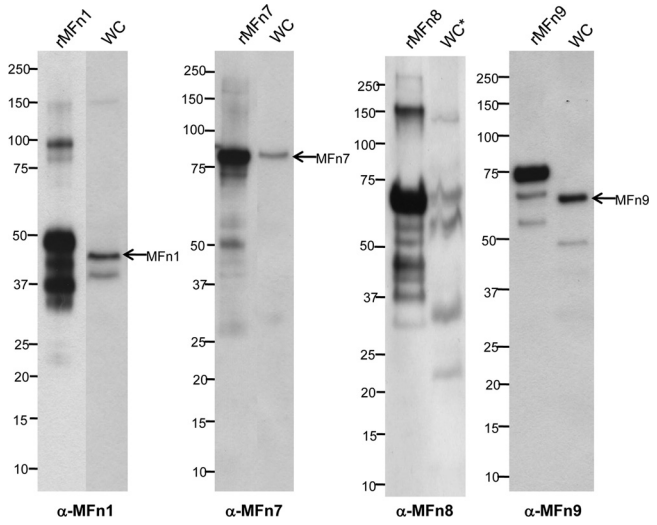

FIG 6 Expression of MFn proteins in cultivated *L. interrogans*. Lanes contain 10^8 leptospires or 0.5 μg of recombinant proteins separated by gel electrophoresis, blotted onto PVDF membrane, and probed with rabbit immune sera specified below each blot. rMFn1, rMFn7, rMFn8, and rMFn9 denote the corresponding recombinant proteins. Lane WC contains *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 whole-cell lysate. The asterisk indicates a 3-fold increase in the amount of whole-cell lysate (3×10^8) loaded into wells. The identities of individual proteins are indicated on the right, and the positions of the molecular mass standards (in kilodaltons) are indicated on the left.

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. 8D and E) or known release of Sph2 to the growth medium (62). Other MFn proteins were not subjected to surface proteolysis due to the 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 an *mfn1*, *mfn3*, *mfn4*, *mfn7*, *mfn9*, or *mfn12* gene was not detected by Coomassie blue staining (data not shown). Therefore, transformants were subjected to further analysis by immunoblotting (Fig. 8) utilizing rabbit serum raised against recombinant proteins or an *L. interrogans* peptide, as described in Materials and Methods. The lack of antibodies recognizing MFn12 prevented the assessment of MFn12 expression by *L. biflexa* transformants. Only the 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 mass (48 kDa) of MFn1 was present only in the *L. biflexa*/MFn1 transformant (Fig. 8A, lane Lb/1). Although *L. bifl-*

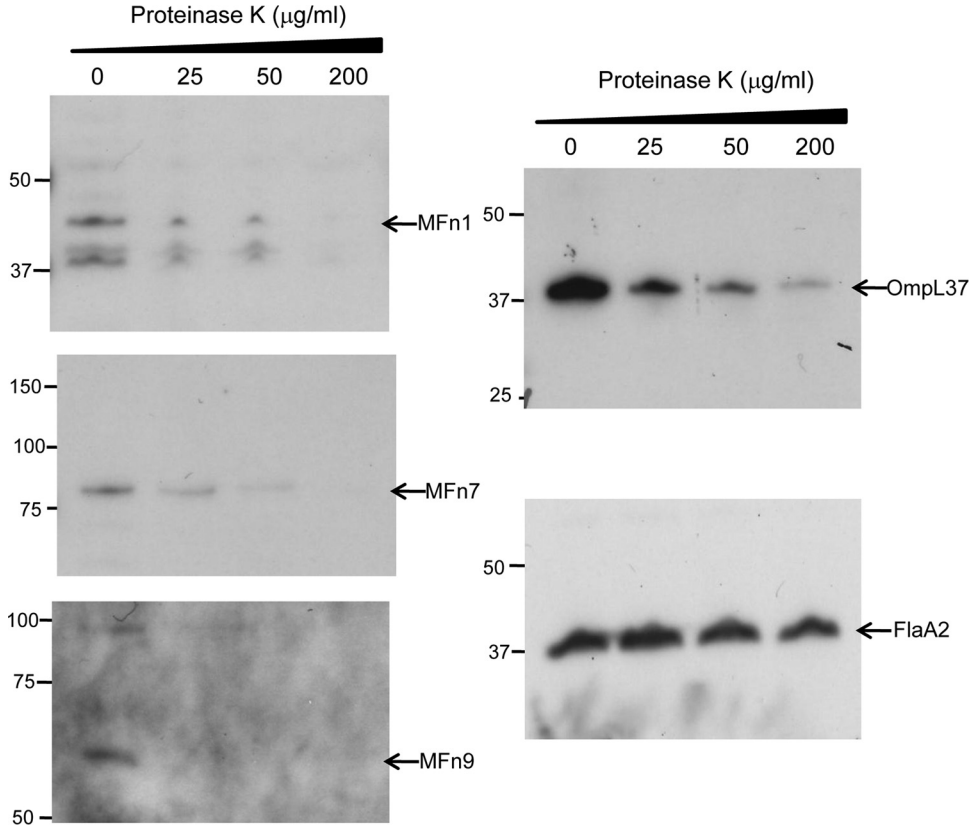

FIG 7 Surface localization of MFn proteins. Intact spirochetes were incubated with different concentrations of proteinase K. Equivalents of 10^8 leptospires per lane were separated by gel electrophoresis, transferred to a PVDF membrane, and probed with polyclonal rabbit antisera against MFn1, MFn7, MFn9, OmpL37, and FlaA2. The identities of individual proteins are indicated on the right, and the positions of the molecular mass standards (in kilodaltons) are indicated on the left.

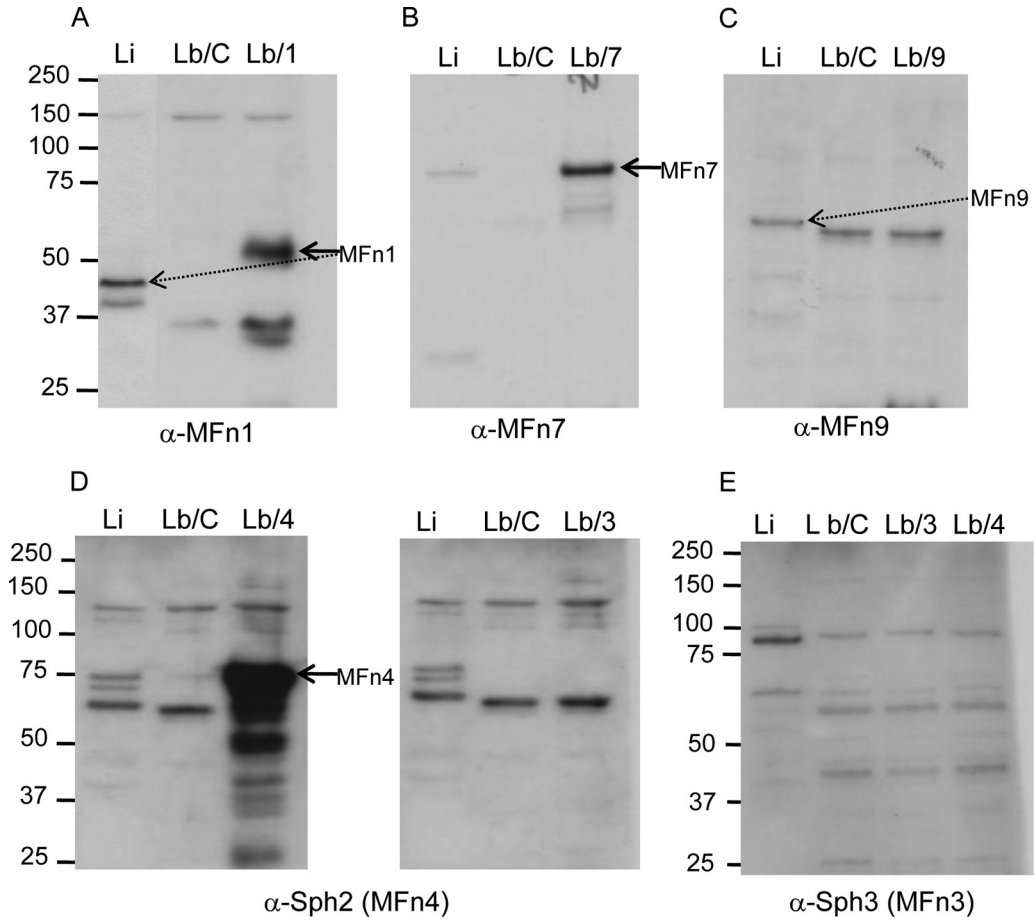

FIG 8 Expression of MFn proteins by *L. biflexa* transformants. Whole-cell lysates of 10^8 leptospires per lane were separated by gel electrophoresis, blotted onto PVDF membrane, and probed with rabbit immune sera recognizing MFn1 (A), MFn7 (B), MFn9 (C), MFn4 (Sph2) (D), and MFn3 (Sph3) (E). Lanes: Li, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130; Lb/C, *L. biflexa* serovar Patoc strain Patoc I transformed with an empty pRAT575 vector, used as a control; Lb/1, Lb/3, Lb/4, Lb/7, and 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.

exa does have a MFn7 homolog (LBF_1774, 32% identity), no expression was detected in the control, *L. biflexa*/pRAT575, whereas the 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% identity), 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) (the present 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 (Fig. 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 nonspecific antibodies present in preimmune serum of a rabbit that was used for immunization with the Sph3 peptide (data not shown). A 76-kDa band corresponding to a predicted molecular mass 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, since 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). A 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 (see 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 mass 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* trans-

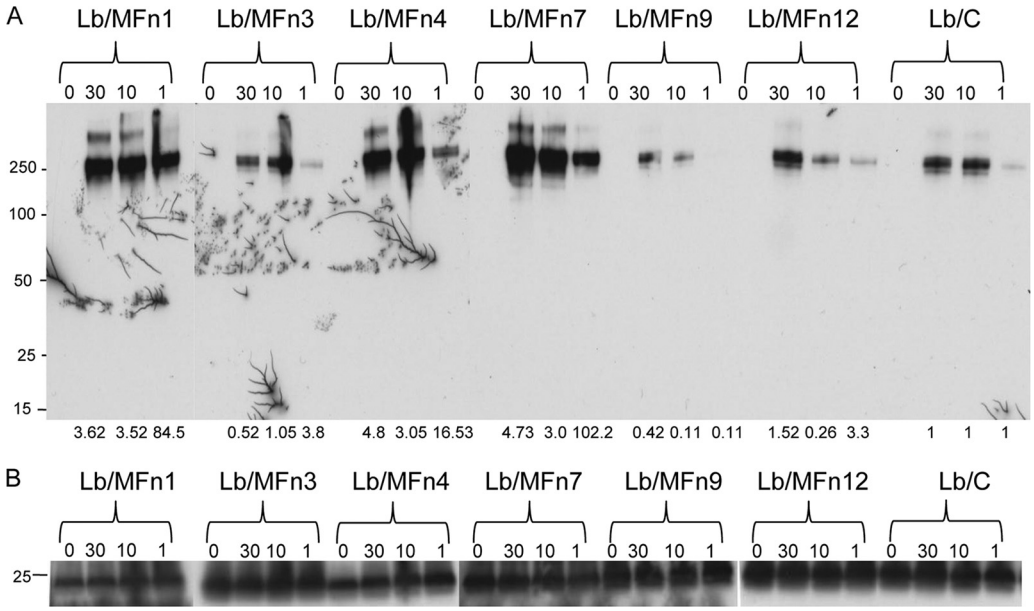

FIG 9 Acquisition of fibronectin by *L. biflexa* transformants. *L. biflexa* transformants were tested for their ability to acquire human plasma fibronectin. After a washing step to remove unbound ligand, 10^8 leptospires per lane were separated by gel electrophoresis, blotted onto PVDF membranes, and 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. The relative densities of the fibronectin bands were standardized to the 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.

formants 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* wild-type strain binds low amounts of fibronectin (31), which is also apparent from our results (Fig. 9). However, densitometry analysis demonstrates that the expression of MFn1, MFn4, and MFn7 dramatically increases binding of fibronectin by *L. biflexa* when supplied at concentrations ranging from 1 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 times more fibronectin 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 or 30 μg/ml was increased to similar levels in *L. biflexa* expressing MFn1, MFn4, and MFn7, ranging from 3.0- to 4.8-fold (Fig. 9A). No substantial increase in fibronectin (10 and 30 μg/ml) binding was observed for *L. biflexa* transformants expressing MFn3, MFn9, or MFn12 (Fig. 9A). Although *L. biflexa* transformants expressing MFn3 and MFn12 increased the binding of fibronectin (1 μg/ml) by 3.8- and 3.3-fold, respectively, this increase was not significant 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 the freely soluble Fn utilized in other assays described here.

DISCUSSION

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 the present 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-

TABLE 3 Summary of OMP microarray-identified fibronectin-binding proteins

MFn protein	Purified rMFn[a]	Antiserum produced	Expression in cultivated *L. interrogans* (size [kDa])	Surface proteolysis	Solid-phase Fn binding[c]	Overexpression in *L. biflexa* transformants	Presence of homologs in *L. biflexa*	Acquisition of Fn by *L. biflexa* transformants
MFn1	D	Whole protein	Yes (48)	Yes	3	Yes	No	Yes
MFn2	D	NA[d]	ND[b]	ND	1	NA	Yes	NA
MFn3 (Sph3)	NA	Synthetic peptide	Yes (90)	ND	ND	No	No	No
MFn4 (Sph2)	NA	N-terminal fragment (amino acids 27 to 190)[f]	Yes (76)	ND	ND	Yes	No	Yes
MFn6	D	NA	ND	ND	4	NA	No	NA
MFn7	D, N	Whole protein	Yes (80)	Yes	2[e]	Yes	Yes	Yes
MFn8	D	Whole protein	No (66)	ND	2	NA	No	NA
MFn9	D	Whole protein	Yes (75)	Yes	5	No	Yes	No
MFn12	NA	NA	ND	ND	ND	ND	Yes	No

[a] D or N, purified under denaturing or native conditions, respectively.
[b] ND, not determined.
[c] Ordered dose-dependent strength of binding: 1 = strongest.
[d] NA, not applicable.
[e] Strongest binding at lower amounts, receptor-like characteristics; only native protein binds.
[f] Data from reference 62.

binding protein FnbpA from *S. aureus*. In these studies, we used the FnbpA-D region that exhibits the highest affinity toward fibronectin (102). Interestingly, of the 15 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 (LfhA = 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 the binding threshold established in our screen. Of all the leptospiral fibronectin-binding proteins studied to date, LigB is the most well characterized, with the highest reported affinity for fibronectin (19, 20, 55, 56), and LigB domains 8 to 12 (20) exhibited considerable fibronectin-binding in our assay (see Table S1 in the supplemental material). Although 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-dependent receptor and TolC family proteins, respectively. Although the 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 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 hydrolyze 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 SphI, 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, noncytotoxic 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 hemolytic 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 9 of 15 leptospiral proteins with MFI values greater than 10 (the results 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 since 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 surfaces 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 immunofluorescence assay data presumed to demonstrate surface exposure of Lsa66 showed a single leptospiral cell with one peripheral fluorescent dot that cannot be dis-

tinguished 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* since 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 the 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). Although 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 a cell-free expression system, which eliminates many of the pitfalls of phage display and Y2H assays, most importantly any 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 of 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 posttranslational protein modifications may adversely affect the identification of functional proteins (64). The lack of posttranslational 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 posttranslational modification may be necessary for the functional activity of some leptospiral OMPs, including adhesins. In fact, it has been proposed that methylation could regulate the switch between an active and an 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 here 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, the 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 the discovery of serodiagnostic antigens is the desired, whole-proteome microarrays would be more beneficial since immunogenic antigens are often subcellular. In addition, 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 14 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 to 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.

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REFERENCES

1. **Abe T, et al.** 2011. OmpA-like protein influences cell shape and adhesive activity of *Tannerella forsythia*. Mol. Oral Microbiol. **26**:374–387.
2. **Adler B, de la Pena Moctezuma A.** 2009. *Leptospira* and leptospirosis. Vet. Microbiol. **140**:287–296.
3. **Athanasopoulos AN, et al.** 2006. The extracellular adherence protein (Eap) of *Staphylococcus aureus* inhibits wound healing by interfering with host defense and repair mechanisms. Blood **107**:2720–2727.
4. **Atzingen MV, et al.** 2008. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. BMC Microbiol. **8**:70. doi:10.1186/1471-2180-8-70.
5. **Atzingen MV, et al.** 2009. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. J. Infect. **59**:264–276.
6. **Bacarese-Hamilton T, Mezzasoma L, Ardizzoni A, Bistoni F, Crisanti A.** 2004. Serodiagnosis of infectious diseases with antigen microarrays. J. Appl. Microbiol. **96**:10–17.
7. **Bagos PG, Liakopoulos TD, Spyropoulos IC, Hamodrakas SJ.** 2004. PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. Nucleic Acids Res. **32**:W400–W404.

8. **Barbosa AS, et al.** 2006. A newly identified leptospiral adhesin mediates attachment to laminin. Infect. Immun. **74**:6356–6364.
9. **Barbour AG, et al.** 2008. A genome-wide proteome array reveals a limited set of immunogens in natural infections of humans and white-footed mice with *Borrelia burgdorferi*. Infect. Immun. **76**:3374–3389.
10. **Bauby H, Saint Girons I, Picardeau M.** 2003. Construction and complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*. Microbiology **149**:689–693.
11. **Bharti AR, et al.** 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. **3**:757–771.
12. **Borrebaeck CA, Wingren C.** 2009. Design of high-density antibody microarrays for disease proteomics: key technological issues. J. Proteomics **72**:928–935.
13. **Bramley AJ, Patel AH, O'Reilly M, Foster R, Foster TJ.** 1989. Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. Infect. Immun. **57**:2489–2494.
14. **Bulach DM, et al.** 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. Proc. Natl. Acad. Sci. U. S. A. **103**:14560–14565.
15. **Cai HY, Lu L, Muckle CA, Prescott JF, Chen S.** 2005. Development of a novel protein microarray method for serotyping *Salmonella enterica* strains. J. Clin. Microbiol. **43**:3427–3430.
16. **Carvalho E, et al.** 2009. Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. FEBS Lett. **583**:1381–1385.
17. **Carvalho E, et al.** 2010. Evaluation of the expression and protective potential of leptospiral sphingomyelinases. Curr. Microbiol. **60**:134–142.
18. **Chirathaworn C, Patarakul K, Saksit V, Poovorawan Y.** 2007. Binding of *Leptospira* to extracellular matrix proteins. J. Med. Assoc. Thai. **90**:2136–2142.
19. **Choy HA, et al.** 2007. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect. Immun. **75**:2441–2450.
20. **Choy HA, et al.** 2011. The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira interrogans*. PLoS One **6**:e16879. doi:10.1371/journal.pone.0016879.
21. **Coburn J, Cugini C.** 2003. Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin αvβ3. Proc. Natl. Acad. Sci. U. S. A. **100**:7301–7306.
22. **Cruz-Fisher MI, et al.** 2011. Identification of immunodominant antigens by probing a whole *Chlamydia trachomatis* open reading frame proteome microarray using sera from immunized mice. Infect. Immun. **79**:246–257.
23. **Cullen PA, Haake DA, Adler B.** 2004. Outer membrane proteins of pathogenic spirochetes. FEMS Microbiol. Rev. **28**:291–318.
24. **Cullen PA, et al.** 2005. Surfaceome of *Leptospira* spp. Infect. Immun. **73**:4853–4863.
25. **Dasgupta G, et al.** 2012. Immunodominant "asymptomatic" herpes simplex virus type 1 and type 2 protein antigens identified by probing whole ORFome microarrays by serum antibodies from seropositive asymptomatic versus symptomatic individuals. J. Virol. **86**:4358–4369.
26. **Davies DH, et al.** 2005. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. Proc. Natl. Acad. Sci. U. S. A. **102**:547–552.
27. **Domingos RF, et al.** 2012. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. BMC Microbiol. **12**:50. doi:10.1186/1471-2180-12-50.
28. **Eshghi A, et al.** 2012. Methylation and *in vivo* expression of the surface-exposed *Leptospira interrogans* outer membrane protein OmpL32. Microbiology **158**:622–635.
29. **Faine S, Adler B, Bolin C, Perolat P.** 1999. *Leptospira* and leptospirosis, 2nd ed. MedSci, Melbourne, Victoria, Australia.
30. **Fields S, Song O.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340**:245–246.
31. **Figueira CP, et al.** 2011. Heterologous expression of pathogen-specific genes *ligA* and *ligB* in the saprophyte *Leptospira biflexa* confers enhanced adhesion to cultured cells and fibronectin. BMC Microbiol. **11**:129. doi:10.1186/1471-2180-11-129.
32. **Galeotti CL, et al.** 2012. Surface interactome in *Streptococcus pyogenes*. Mol. Cell. Proteomics **11**:M111.015206.
33. **Gamberini M, et al.** 2005. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol. Lett. **244**:305–313.
34. **Ganoza CA, et al.** 2010. Asymptomatic renal colonization of humans in the Peruvian Amazon by leptospira. PLoS Negl. Trop. Dis. **4**:e612. doi:10.1371/journal.pntd.0000612.
35. **Girons IS, et al.** 2000. The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: construction of an *L. biflexa-Escherichia coli* shuttle vector. J. Bacteriol. **182**:5700–5705.
36. **Gonzalez-Zorn B, et al.** 1999. The *smcL* gene of *Listeria ivanovii* encodes a sphingomyelinase C that mediates bacterial escape from the phagocytic vacuole. Mol. Microbiol. **33**:510–523.
37. **Gromiha MM, Ahmad S, Suwa M.** 2005. TMBETA-NET: discrimination and prediction of membrane spanning beta-strands in outer membrane proteins. Nucleic Acids Res. **33**:W164–W167.
38. **Haake DA.** 2000. Spirochaetal lipoproteins and pathogenesis. Microbiology **146**(Pt 7):1491–1504.
39. **Hartford OM, Wann ER, Hook M, Foster TJ.** 2001. Identification of residues in the *Staphylococcus aureus* fibrinogen-binding MSCRAMM clumping factor A (ClfA) that are important for ligand binding. J. Biol. Chem. **276**:2466–2473.
40. **Hartskeerl RA, Collares-Pereira M, Ellis WA.** 2011. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin. Microbiol. Infect. **17**:494–501.
41. **Hauk P, et al.** 2008. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. Infect. Immun. **76**:2642–2650.
42. **Hermanson G, et al.** 2012. Measurement of antibody responses to modified vaccinia virus Ankara (MVA) and Dryvax® using proteome microarrays and development of recombinant protein ELISAs. Vaccine **30**:614–625.
43. **Hoke DE, Egan S, Cullen PA, Adler B.** 2008. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. Infect. Immun. **76**:2063–2069.
44. **Hoppe S, Bier FF, von Nickisch-Rosenegk M.** 2012. Microarray-based method for screening of immunogenic proteins from bacteria. J. Nanobiotechnol. **10**:12.
45. **Huseby M, et al.** 2007. Structure and biological activities of beta toxin from *Staphylococcus aureus*. J. Bacteriol. **189**:8719–8726.
46. **Ito T, Yanagawa R.** 1987. Leptospiral attachment to four structural components of extracellular matrix. Nippon Juigaku Zasshi **49**:875–882.
47. **Juncker AS, et al.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. **12**:1652–1662.
48. **Keasey SL, et al.** 2009. Extensive antibody cross-reactivity among infectious gram-negative bacteria revealed by proteome microarray analysis. Mol. Cell. Proteomics **8**:924–935.
49. **Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD, Jr, Riley LW.** 1999. Urban epidemic of severe leptospirosis in Brazil. Lancet **354**:820–825.
50. **Koebnik R, Locher KP, Van Gelder P.** 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. **37**:239–253.
51. **Korf U, Wiemann S.** 2005. Protein microarrays as a discovery tool for studying protein-protein interactions. Expert Rev. Proteomics **2**:13–26.
52. **Lee SH, Kim S, Park SC, Kim MJ.** 2002. Cytotoxic activities of *Leptospira interrogans* hemolysin SphH as a pore-forming protein on mammalian cells. Infect. Immun. **70**:315–322.
53. **Levett, P. N.** 2001. Leptospirosis. Clin. Microbiol. Rev. **14**:296–326.
54. **Li B, et al.** 2005. Protein microarray for profiling antibody responses to *Yersinia pestis* live vaccine. Infect. Immun. **73**:3734–3739.
55. **Lin YP, Chang YF.** 2007. A domain of the *Leptospira* LigB contributes to high-affinity binding of fibronectin. Biochem. Biophys. Res. Commun. **362**:443–448.
56. **Lin YP, et al.** 2009. Repeated domains of *Leptospira* immunoglobulin-like proteins interact with elastin and tropoelastin. J. Biol. Chem. **284**:19380–19391.
57. **Longhi MT, et al.** 2009. A newly identified protein of *Leptospira interrogans* mediates binding to laminin. J. Med. Microbiol. **58**:1275–1282.
58. **Louvel H, Picardeau M.** 2007. Genetic manipulation of *Leptospira biflexa*. Curr. Protoc. Microbiol. **Chapter 12**:Unit 12E14.
59. **Luevano M, et al.** 2010. High-throughput profiling of the humoral immune responses against thirteen human papillomavirus types by proteome microarrays. Virology **405**:31–40.

60. **MacBeath G, Schreiber SL.** 2000. Printing proteins as microarrays for high-throughput function determination. Science **289**:1760–1763.
61. **Margarit I, et al.** 2009. Capturing host-pathogen interactions by protein microarrays: identification of novel streptococcal proteins binding to human fibronectin, fibrinogen, and C4BP. FASEB J. **23**:3100–3112.
62. **Matsunaga J, Medeiros MA, Sanchez Y, Werneid KF, Ko AI.** 2007. Osmotic regulation of expression of two extracellular matrix-binding proteins and a haemolysin of *Leptospira interrogans*: differential effects on LigA and Sph2 extracellular release. Microbiology **153**:3390–3398.
63. **McBride AJ, Athanazio DA, Reis MG, Ko AI.** 2005. Leptospirosis. Curr. Opin. Infect. Dis. **18**:376–386.
64. **McCafferty J.** 1996. Phage display: factors affecting panning efficiency. *In* Kay JWBK, McCafferty J (ed), Phage display of peptides and proteins: a laboratory manual. Academic Press, Inc, San Diego, CA.
65. **Mendes RS, et al.** 2011. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. Infect. Immun. **79**:4657–4667.
66. **Mendez-Rios J, Uetz P.** 2010. Global approaches to study protein-protein interactions among viruses and hosts. Future Microbiol. **5**:289–301.
67. **Merien F, Truccolo J, Baranton G, Perolat P.** 2000. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar icterohaemorrhagiae. FEMS Microbiol. Lett. **185**:17–22.
68. **Molina DM, et al.** 2010. Identification of immunodominant antigens of *Chlamydia trachomatis* using proteome microarrays. Vaccine **28**:3014–3024.
69. **Mulvey MA.** 2002. Adhesion and entry of uropathogenic *Escherichia coli*. Cell Microbiol. **4**:257–271.
70. **Narayanavari SA, Kishore NM, Sritharan M.** 2012. Structural analysis of the leptospiral sphingomyelinases: *in silico* and experimental evaluation of Sph2 as an Mg-dependent sphingomyelinase. J. Mol. Microbiol. Biotechnol. **22**:24–34.
71. **Narayanavari SA, Sritharan M, Haake DA, Matsunaga J.** 2012. Multiple leptospiral sphingomyelinases (or are there?). Microbiology **158**:1137–1146.
72. **Nascimento AL, et al.** 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J. Bacteriol. **186**:2164–2172.
73. **Natesan M, Ulrich RG.** 2010. Protein microarrays and biomarkers of infectious disease. Int. J. Mol. Sci. **11**:5165–5183.
74. **Nielsen H, Engelbrecht J, Brunak S, von Heijne G.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**:1–6.
75. **Oliveira R, et al.** 2011. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. PLoS One **6**:e21962. doi:10.1371/journal.pone.0021962.
76. **Oliveira TR, et al.** 2010. LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. Microbes Infect. **12**:207–217.
77. **Patti JM, Allen BL, McGavin MJ, Hook M.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu. Rev. Microbiol. **48**:585–617.
78. **Picardeau M, et al.** 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One **3**:e1607. doi:10.1371/journal.pone.0001607.
79. **Pinne M, Choy HA, Haake DA.** 2010. The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. PLoS Negl. Trop. Dis. **4**:e815. doi:10.1371/journal.pntd.0000815.
80. **Pinne M, Haake DA.** 2009. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. PLoS One **4**:e6071. doi:10.1371/journal.pone.0006071.
81. **Pinne M, et al.** 2007. Elimination of channel-forming activity by insertional inactivation of the p66 gene in *Borrelia burgdorferi*. FEMS Microbiol. Lett. **266**:241–249.
82. **Pizarro-Cerda J, Cossart P.** 2006. Bacterial adhesion and entry into host cells. Cell **124**:715–727.
83. **Ponnuraj K, et al.** 2003. A "dock, lock, and latch" structural model for a staphylococcal adhesin binding to fibrinogen. Cell **115**:217–228.
84. **Provenzano D, Klose KE.** 2000. Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization. Proc. Natl. Acad. Sci. U. S. A. **97**:10220–10224.
85. **Ramachandran N, Srivastava S, Labaer J.** 2008. Applications of protein microarrays for biomarker discovery. Proteomics Clin. Applications **2**:1444–1459.
86. **Ren SX, et al.** 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature **422**:888–893.
87. **Rentero I, Heinis C.** 2011. Screening of large molecule diversities by phage display. Chimia **65**:843–845.
88. **Schulz G.** 2004. The structures of general porins, p 25–40. *In* Benz R (ed), Bacterial and eukaryotic porins: structure, function, and mechanism. Wiley-VCH Verlag GmbH & Co, KGaA, Weinheim, Germany.
89. **Setubal JC, Reis M, Matsunaga J, Haake DA.** 2006. Lipoprotein computational prediction in spirochaetal genomes. Microbiology **152**:113–121.
90. **Smith GP.** 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science **228**:1315–1317.
91. **Steller S, et al.** 2005. Bacterial protein microarrays for identification of new potential diagnostic markers for *Neisseria meningitidis* infections. Proteomics **5**:2048–2055.
92. **Stevenson B, et al.** 2007. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. PLoS One **2**:e1188. doi:10.1371/journal.pone.0001188.
93. **Sundaresh S, et al.** 2006. Identification of humoral immune responses in protein microarrays using DNA microarray data analysis techniques. Bioinformatics **22**:1760–1766.
94. **Suwannasaen D, et al.** 2011. Human immune responses to *Burkholderia pseudomallei* characterized by protein microarray analysis. J. Infect. Dis. **203**:1002–1011.
95. **Tomizaki KY, Usui K, Mihara H.** 2010. Protein-protein interactions and selection: array-based techniques for screening disease-associated biomarkers in predictive/early diagnosis. FEBS J. **277**:1996–2005.
96. **Trevejo RT, et al.** 1998. Epidemic leptospirosis associated with pulmonary hemorrhage–Nicaragua, 1995. J. Infect. Dis. **178**:1457–1463.
97. **Verma A, et al.** 2006. LfhA, a novel factor H-binding protein of *Leptospira interrogans*. Infect. Immun. **74**:2659–2666.
98. **Vieira ML, et al.** 2009. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. J. Infect. **60**:52–64.
99. **Vigil A, et al.** 2011. Profiling the humoral immune response of acute and chronic Q fever by protein microarray. Mol. Cell. Proteomics **10**:M110 006304.
100. **Vigil A, Davies DH, Felgner PL.** 2010. Defining the humoral immune response to infectious agents using high-density protein microarrays. Future Microbiol. **5**:241–251.
101. **Vigil A, et al.** 2010. Genome-wide profiling of humoral immune response to *Coxiella burnetii* infection by protein microarray. Proteomics **10**:2259–2269.
102. **Wann ER, Gurusiddappa S, Hook M.** 2000. The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. J. Biol. Chem. **275**:13863–13871.
103. **Xu Y, Rivas JM, Brown EL, Liang X, Hook M.** 2004. Virulence potential of the staphylococcal adhesin CNA in experimental arthritis is determined by its affinity for collagen. J. Infect. Dis. **189**:2323–2333.
104. **Young KT, Davis LM, Dirita VJ.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat. Rev. Microbiol. **5**:665–679.
105. **Zhang YX, Yang GYJW, Guo XK, Zhao GP.** 2008. Cytotoxic activity and probable apoptotic effect of Sph2, a sphingomyelinase hemolysin from *Leptospira interrogans* serovar Lai. BMB Rep. **41**:119–125.
106. **Zhu H, Bilgin M, Snyder M.** 2003. Proteomics. Annu. Rev. Biochem. **72**:783–812.
107. **Zhu H, et al.** 2006. Severe acute respiratory syndrome diagnostics using a coronavirus protein microarray. Proc. Natl. Acad. Sci. U. S. A. **103**:4011–4016.
108. **Zilbauer M, Dorrell N, Wren BW, Bajaj-Elliott M.** 2008. *Campylobacter jejuni*-mediated disease pathogenesis: an update. Trans. R. Soc. Trop. Med. Hyg. **102**:123–129.