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Expression and Characterization of an Iron-Regulated Hemin-Binding Protein, HbpA, from *Leptospira interrogans* Serovar Lai[▽]

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In an earlier study, based on the ferric enterobactin receptor FepA of *Escherichia coli*, we identified and modeled a TonB-dependent outer membrane receptor protein (LB191) from the genome of *Leptospira interrogans* serovar Lai. Based on in silico analysis, we hypothesized that this protein was an iron-dependent hemin-binding protein. In this study, we provide experimental evidence to prove that this protein, termed HbpA (*h*emin-*b*inding *p*rotein A), is indeed an iron-regulated hemin-binding protein. We cloned and expressed the full-length 81-kDa recombinant rHbpA protein and a truncated 55-kDa protein from *L. interrogans* serovar Lai, both of which bind hemin-agarose. Assay of hemin-associated peroxidase activity and spectrofluorimetric analysis provided confirmatory evidence of hemin binding by HbpA. Immunofluorescence studies by confocal microscopy and the microscopic agglutination test demonstrated the surface localization and the iron-regulated expression of HbpA in *L. interrogans*. Southern blot analysis confirmed our earlier observation that the *hbpA* gene was present only in some of the pathogenic serovars and was absent in *Leptospira biflexa*. Hemin-agarose affinity studies showed another hemin-binding protein with a molecular mass of approximately 44 kDa, whose expression was independent of iron levels. This protein was seen in several serovars, including nonpathogenic *L. biflexa*. Sequence analysis and immunoreactivity with specific antibodies showed this protein to be LipL41.

Leptospirosis is a zoonotic disease and has a worldwide distribution (2). Humans are accidental hosts, in whom the disease can manifest itself with a wide range of clinical symptoms ranging from subclinical infection to undifferentiated febrile illness, jaundice, renal failure, and potentially lethal pulmonary hemorrhage. There is a lack of fundamental understanding of several aspects of the biology of *Leptospira* spp. and, more importantly, of the adaptation of these pathogens to the hostile environment prevailing within the mammalian host. The whole-genome sequence data (26, 28) will aid in better understanding the host-pathogen interrelationship and pathogenesis of this disease. One of the factors playing an important role in pathogenesis, as understood for several bacterial systems (11, 31), is iron acquisition.

Iron is required for the growth of nearly all organisms and is an essential cofactor of numerous metabolic and enzymatic processes (10). Low solubility of the ferric iron at biological pH coupled with the sequestering of iron as a part of the innate immune system of the mammalian host restricts the availability of free iron to the invading microorganisms. Pathogenic bacteria, however, have adapted to this iron-restricted environment prevailing within the mammalian host and express unique iron acquisition systems (4). Siderophore-mediated iron uptake is commonly seen in several bacteria, while others, including *Neisseria* and *Pasteurella* species, express specific outer membrane receptors that chelate the iron from host iron-containing molecules such as transferrin, lactoferrin, and heme compounds (27, 30). Since greater than 90% of the iron within the human body is associated with heme and heme-containing proteins, bacteria that can access these compounds and utilize the heme iron have a significant nutritional advantage. *Vibrio cholerae* (15, 34), enterohemorrhagic *Escherichia coli* O157:H7 (36), *Shigella dysenteriae* (25), *Yersinia pestis* (16), and *Yersinia enterocolitica* (35) are some examples of bacterial pathogens that produce TonB-dependent outer membrane receptors that bind hemin, which is subsequently internalized with the help of ATP-binding cassette (ABC) transporters. A second type of heme uptake system, identified in certain species such as *Serratia marcescens* (3) and *Pseudomonas aeruginosa* (21), involves the secretion of heme-binding proteins called hemophores that bind heme and transport it to the cell surface to be internalized by specific cell surface receptors. In either of the systems, the hemin can either be internalized as such or the iron alone can be internalized after it is released from the hemin at the cell surface (5). In addition, the association of iron with the expression of virulence factors is well known in several bacterial systems (11, 31, 33).

Iron is an essential nutrient for pathogenic leptospires (9). Louvel et al. (23) performed random insertional mutagenesis with the saprophytic *Leptospira biflexa* and identified five hemin-requiring mutants. Three of these mutants had insertions in a gene encoding a protein that shares homology with the TonB-dependent ferric citrate receptor FecA of *E. coli*, and the other two mutants showed a *Himar1* insertion into a *feoB*-like gene; FeoB plays a role in ferrous iron uptake in several bacteria. In a recent report (24), they discuss their observations on iron acquisition in *L. biflexa* in light of the data obtained from the whole-genome sequencing. Cullen et al. (7), in a detailed analysis of the outer membrane proteins of *Leptospira*

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interrogans serovar Lai maintained under different growth conditions, showed that LipL32, LipL36, pL50, and pL24 were influenced by both temperature and iron.

Efforts in our lab to understand iron acquisition in leptospires included the identification (LB191; GenBank accession number AE011607) and modeling of a putative TonB-dependent outer membrane receptor protein (32), which, despite showing low levels of similarity (39%) and identity (22%) with FepA of *E. coli*, revealed features of protein folding like those of other Fe^{3+}-siderophore/hemin receptors. The putative Fur box located upstream of LB191 and the presence of LB183 (*fur* gene encoding the Fur regulator) and LB186 (encoding heme oxygenase) led us to hypothesize that this protein is an iron-regulated hemin-binding protein. We henceforth refer to this protein as HbpA (*h*emin-*b*inding *p*rotein A).

In this study, we provide experimental evidence to confirm our hypothesis and show that the 81-kDa HbpA in *L. interrogans* serovar Lai binds hemin and is expressed upon iron deprivation. In addition, we identified another constitutively expressed hemin-binding protein with a molecular mass of approximately 44 kDa whose expression was independent of iron levels. This protein, expressed by several leptospiral serovars, was found to be LipL41 by sequencing and immunoblotting with specific anti-LipL41 antibodies.

MATERIALS AND METHODS

Strains and growth conditions. The leptospiral serovars used in this study were obtained from the National Repository at the Regional Medical Research Centre, ICMR, Port Blair, Andaman and Nicobar Islands, India. The *E. coli* strains included DH5α (lab collection) and BL21(DE3)/pLysS (Novagen).

Leptospires were maintained in 0.2% agar-containing semisolid EMJH medium supplemented with 10% enrichment medium (Difco) at 30°C. The cells were regularly grown in liquid EMJH medium (the concentration of iron was 10 μg/ml) for about 10 days, and cells in the log phase were used for growth under high- and low-iron conditions (as detailed below).

The *E. coli* strains were routinely grown in Luria-Bertani (LB) medium at 37°C, with ampicillin (50 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (34 μg/ml) added as required for the appropriate strains.

Chromosomal DNA isolation and Southern blot analysis. Chromosomal DNA was isolated according to standard protocols (29). For Southern blot analysis, 10 μg of chromosomal DNA was digested with HindIII overnight at 37°C, followed by transfer to Nylon$^+$ membrane (Amersham) by vacuum blotting. The blot was UV cross-linked for 1.4 min, incubated in prehybridization buffer (0.5 M phosphate buffer [pH 7.0] containing 7% sodium dodecyl sulfate [SDS], 1 mM EDTA, and 100 mg bovine serum albumin [BSA]) for 4 h at 65°C. DNA probe (100 ng of PCR amplified *hbpA*) labeled with [α-^{32}P]dATP by random primer DNA labeling was denatured at 100°C and quickly chilled in ice. This was added to the blot in prehybridization buffer, and hybridization was done for 16 h at 65°C. After unbound probe was washed, the blot was subjected to autoradiography.

PCR amplification, cloning, and expression studies. Using genomic DNA of *L. interrogans* serovar Lai as a template, the full-length 2,148-bp *hbpA* gene was amplified with primers L1 (with an NdeI site; 5′ GGG AAT TCC ATA TGT CAT CCA ACC ATT CGA TG 3′) and L2 (with an Hind III site; 5′ CCC AAG CTT TTA AAA GTG GGC CGA GAA TC 3′), using an initial denaturation for 5 min (95°C) followed by 30 cycles of amplification (1 min at 95°C, 1 min at 50°C, and 2.3 min at 72°C) and a final extension for 30 min at 72°C done in a PTC-200 thermal cycler (MJ Research). The PCR product was cloned by standard protocols (29). It was initially cloned into the TA vector and then recloned into pET28a(+) (Novagen). Transformation was initially done in *E. coli* DH5α and then into BL21(DE3)/BL21(DE3)/pLysS (Novagen) for expression studies. Colonies were screened by colony PCR for the identification of positive clones.

The sequence of one of the positive clones was confirmed in the Megabase 500 automated DNA sequencer (Amersham) by using the DYEnamic ET dye terminator cycle sequencing kit for MegaBace (This was done in the laboratory of A. R. Reddy, University of Hyderabad).

Expression, purification, and raising of antibodies against recombinant HbpA (rHbpA) were done as follows. One millimolar IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma) was added to a growing culture (optical density at 600 nm [OD_{600}] of 0.6) of a positive clone. Samples were collected at hourly intervals and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel (18). HbpA was purified with the Bug Buster Ni-nitrilotriacetic acid (NTA) His-Bind purification kit (Novagen). Anti-HbpA antibodies were raised by immunizing rabbits with 100 μg of the purified protein in Freund's complete adjuvant, followed by a booster after 3 weeks (40 μg).

We expressed a truncated $rHbpA_{55}$ that was obtained by cloning and expression of an 1,449-bp fragment representing *hbpA* lacking 683 bp from the 5′ end. This 1,449-bp fragment was amplified with primers L3 (with an NdeI site; GGG AAT TCC ATA TGG AAT TCA ATA CCA CAG CCA ACA TGG G) and L2 (shown above). It was cloned into the pET28(a) vector and transformed into BL(DE3) for expression studies.

Hemin-binding studies with HbpA. HbpA was assayed for its hemin-binding ability in three separate experiments, including binding to hemin-agarose beads, spectrofluorimetric analysis, and assaying the peroxidase activity of the protein-bound hemin.

Binding to hemin-agarose was performed essentially as described by Lee (19). Briefly, 200 μl of hemin-agarose (Sigma-Aldrich) was washed thrice in 1 ml of 100 mM NaCl–25 mM Tris-HCl (pH 7.4), with centrifugation done at 750 × *g* for 5 min. In two independent experiments, hemin-agarose was incubated with column-purified HbpA (20 μg) and the crude cell lysate of recombinant *E. coli* solubilized in Bug Buster reagent (total of 1 mg protein in 1 ml) for 1 h at 37°C with gentle mixing. After three washes to remove unbound proteins, the hemin-agarose beads were incubated for 2 min with 2% (wt/vol) SDS and 1% (vol/vol) β-mercaptoethanol in 500 mM Tris HCl (pH 6.8), boiled at 100°C for 5 min, and centrifuged and the supernatant was subjected to SDS-PAGE on a 10% gel.

Spectrofluorimetric analysis, performed in SPEX Fluoromax-3 fluorescence spectrophotometer (Jobin-Yvon, Edison, NJ), was done with purified HbpA at an OD_{280} of approximately 0.05. The sample was excited with light with a wavelength of 295 nm, and the emission spectrum was recorded at 300 nm and above; slit widths of 3 and 6 nm for excitation and emission, respectively, were used, and the integration time was set at 0.3 s. Suitable aliquots of a 1.5 mM solution of hemin were added and recordings taken. The fluorescence intensities were corrected for volume changes before further analysis of the quenching data. All measurements were performed at 25°C.

For the assay of peroxidase activity of the protein-bound hemin, a microtiter plate was coated with HbpA at various protein concentrations and incubated with hemin (20 μg/100 μl) at 37°C for 1 h. The unbound hemin was removed, wells were washed thrice with phosphate-buffered saline (PBS) (0.01 M, pH 7), and 100 μl of the ready-to-use substrate tetramethylbenzidine/H_2O_2 (Bangalore-Genei, India) was added. After incubation for 20 min with the substrate, the reaction was stopped with 1 N H_2SO_4 and the OD_{450} was determined in an enzyme-linked immunosorbent assay reader. The amount of hemin bound to HbpA was calculated from the peroxidase activities of known concentrations of hemin.

Iron-regulated expression of HbpA. Growth of *L. interrogans* serovar Lai in high- and low-iron media was done as follows. The log-phase culture in liquid EMJH medium was scaled up slowly over a period of 10 days with regular EMJH medium plus enrichment medium and then divided equally between two flasks to represent high and low iron levels, respectively. A 100 μM concentration of ethylene diamine-*N*,*N*′-diacetic acid (EDDA) (Sigma) was added to the low-iron flask and incubated for 24 h, and then the EDDA concentration was increased to 200 μM (final concentration) and incubated for another 24 h. During all the additions, the cultures were maintained at 30°C. The cells were then pelleted by centrifugation and resuspended in iron-free medium (EMJH medium with 2% BSA and without iron) that was preincubated overnight with 200 μM EDDA, using iron-free glassware. The volume correction for the high-iron culture was made by the addition of EMJH medium with 2% BSA (containing 10 μg Fe/ml) at the same time periods as for low-iron growth. These organisms were incubated for 5 h at 37°C. The organisms were harvested, washed, and sonicated for 5 min (30-s pulses at 12 Hz in a Vibra Cell sonicator). The cell-free sonicates were then analyzed by SDS-PAGE on a 5 to 20% gradient gel. The proteins were stained with Coomassie brilliant blue R-250 (Sigma). Western blotting analysis of these proteins was done by transfer of the proteins to nitrocellulose (37), incubation with anti-HbpA antibodies (dilution of 1:600), and addition of secondary antibody conjugated to alkaline phosphatase. The blot was developed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (ready-to-use solution from Bangalore Genei, India). An identical experiment was done with *Leptospira borgpetersenii* serovar Tarassovi.

Agglutination of low-iron organisms in the presence of anti-HbpA antibodies. Anti-HbpA antibodies were added to iron-replete and iron-deficient leptospires at dilutions of 1:100 and 1:50 and incubated for 2 h at 37°C in a microtiter plate

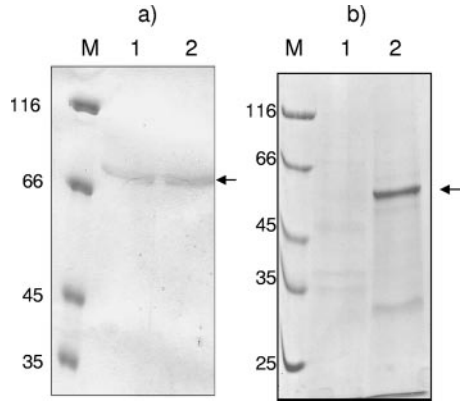

FIG. 1. Hemin-agarose binding of rHbpA and rHbpA$_{55}$. The 81-kDa rHbpA and the 55-kDa rHbpA$_{55}$ bound by hemin-agarose beads are shown as Coomassie blue-stained proteins in panels a and b, respectively. (a) Two independent experiments with hemin-agarose beads, first with incubation with the Ni-NTA column-purified rHbpA (lane 1) and second with incubation with the whole-cell sonicate of the IPTG-induced recombinant clone (lane 2). The 81-kDa rHbpA is indicated by the arrow. (b) Hemin-agarose-bound 55-kDa rHbpA$_{55}$ from whole-cell sonicate of IPTG-induced recombinant clone (lane 2) and uninduced *E. coli* sonicate (lane 1).

as done for microscopic agglutination test assays (100 μl of the cell suspension adjusted to McFarland standard of 1 was used). The agglutination was observed by dark-field microscopy and the images recorded using the Nikon Eclipse E600 (Japan). The preimmune serum was used as the antibody control.

Confocal microscopy and immunofluorescence studies. Confocal microscopy was performed with a Leica TCS SP2 AOBS instrument, based on the published protocol for LipL32 (8). Briefly, *L. interrogans* cells, grown under high- and low-iron conditions (as described above) were harvested at 10,000 rpm for 20 min, washed thrice with PBS (pH 7.2), and resuspended in PBS with 2.5% BSA to a cell density equivalent to McFarland standard of 2. A thin smear was prepared on a slide, allowed to air dry, and then heat fixed by quickly passing the slide through the flame of a Bunsen burner twice. The smear was then treated with ice-cold methanol at −20°C for 40 min and then blocked with 5% BSA for 1.5 h.

Anti-HbpA immunoglobulins were prepared by ammonium sulfate precipitation (followed by dialysis to remove the ammonium sulfate) of polyclonal rabbit antiserum raised against rHbpA using standard protocols. This was added at a dilution of 1:100 to the fixed leptospires and incubated overnight at 4°C. After four washes with PBS with 0.05% Tween 20, the slide was incubated with a 1:500 dilution of goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate (Bangalore Genei) for 1.5 h at room temperature and then subjected to four washes with PBS to remove the unbound conjugate. Propidium iodide (10 μg/ml) was applied as a counterstain. A drop of 90% glycerol was added to the slide to keep it moist, overlaid with a coverslip, and sealed and then the fluorescence was visualized in the confocal microscope.

Identical smear preparations of high- and low-iron organisms from the same batch of cells described above were incubated with anti-LipL41 antibodies. The excitation wavelength for FITC is 500 nm, with emission at 535 nm, while the excitation and emission wavelengths for propidium iodide are 600 and 732 nm, respectively.

Identification, purification, and sequence analysis of a 44-kDa hemin-binding outer membrane protein. The outer membranes of *L. interrogans* serovar Lai strain Lai from both high- and low-iron cells were prepared by published protocols (14). Briefly, the organisms, harvested by centrifugation at 10,000 rpm for 20 min, were washed thrice with 50 mM Tris (pH 8.0) with 5 mM $MgCl_2$ and then incubated overnight at 4°C in 2% Triton X-114 in 10 mM Tris (pH 8.0) containing 1 mM EDTA and 150 mM NaCl. The insoluble material was removed by

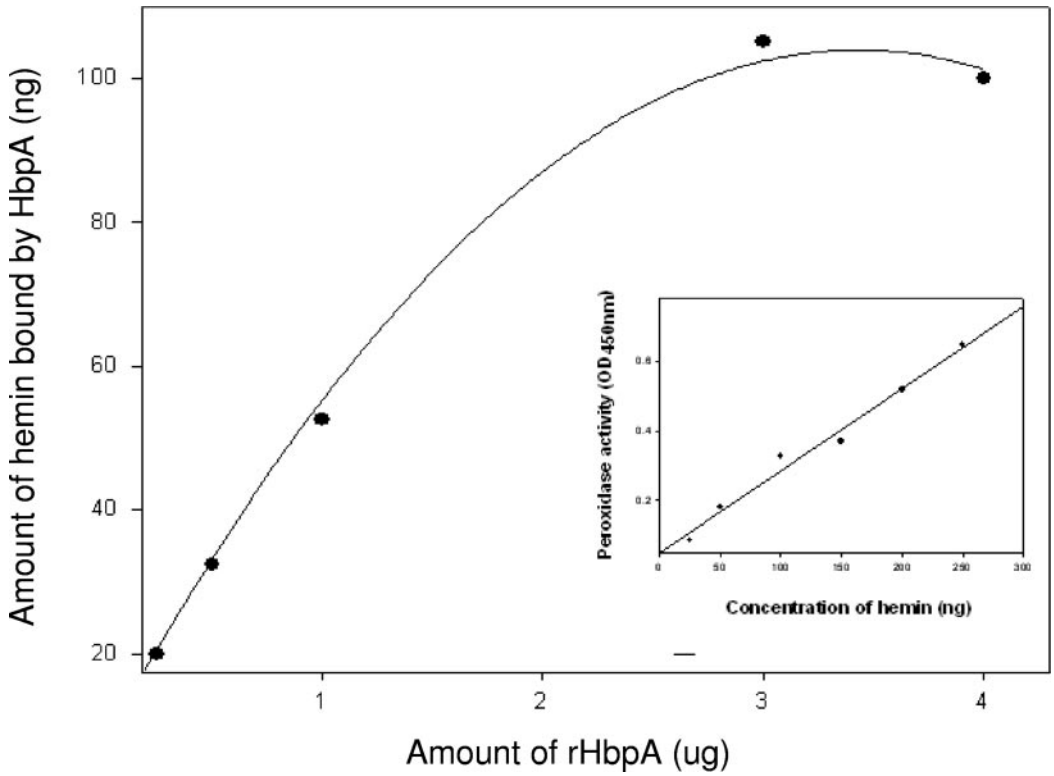

FIG. 2. Peroxidase activity of the hemin bound by HbpA. A microtiter plate was coated with various protein concentrations of HbpA and incubated with 20 μg of hemin in a total volume of 100 μl in a microtiter plate. After suitable washes, the peroxidase activity of the bound hemin was assayed by the addition of tetramethylbenzidine and reading the absorbance at 450 nm. The inset shows the standard graph of peroxidase activity of hemin.

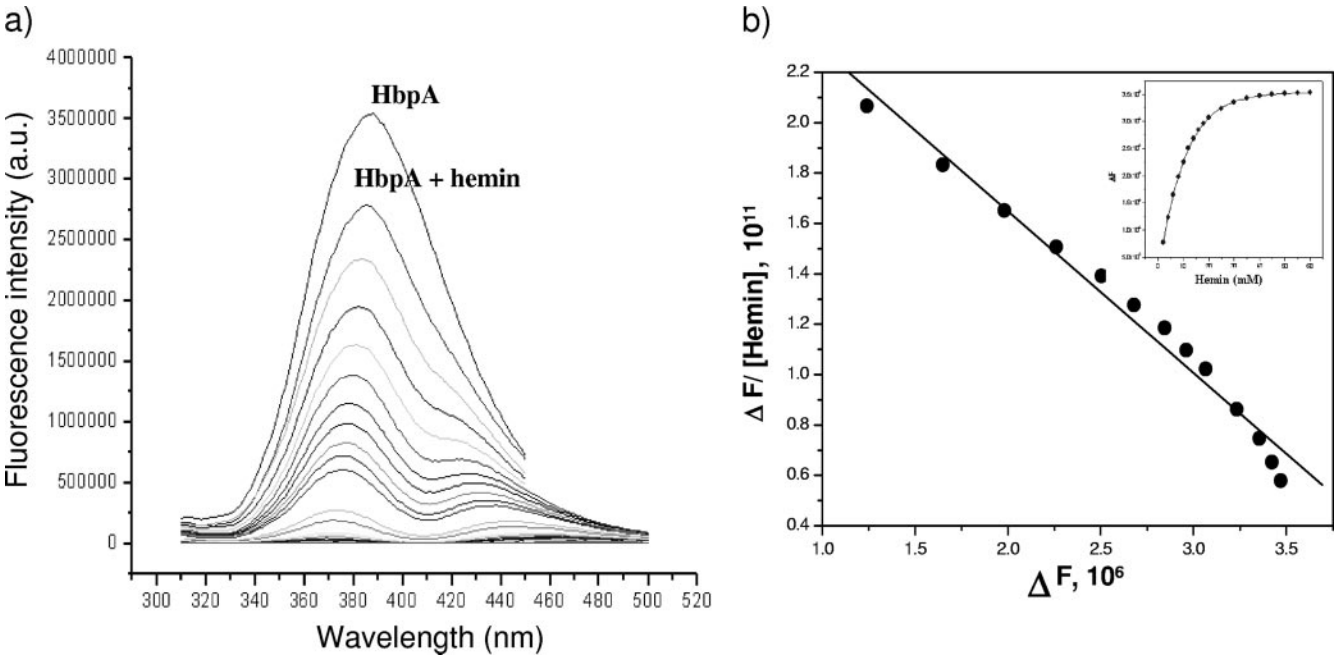

FIG. 3. Spectrofluorimetric analysis of hemin binding by HbpA. (a) Emission spectrum of HbpA upon excitation with light with a wavelength of 295 nm. Hemin (1.5 mM stock) was added at increasing concentrations and the spectrum recorded after each addition. (b) Scatchard plot for the determination of the association constant for hemin binding by HbpA. The inset shows the decrease of fluorescence intensity as a function of hemin concentration. ΔF represents the difference in the values of the emitted spectra upon addition of various amounts of hemin.

centrifugation at 17,000 × *g* for 10 min, and $CaCl_2$ (20 mM) was added to the supernatant. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation at 2,000 × *g* for 15 min. The outer membrane proteins in the detergent phase were precipitated with acetone, subjected to three washes, solubilized, and used for hemin-binding studies. The outer membrane proteins were incubated with hemin-agarose using the protocol described in detail above (for rHbpA) and were analyzed by SDS-PAGE.

The prominent 44-kDa protein from high-iron cells was purified with hemin-agarose beads and subjected to SDS-PAGE, and the purified band was cut out and sequenced by tandem mass spectrometry in the lab of F. H. Friedman (Universitaet fuer Bodenkultur, Austria) using published protocols (40).

Immunoreactivity of the 44-kDa protein with anti-LipL41 antibodies. The 44-kDa hemin-agarose purified protein was tested by immunoblotting with anti-LipL41 antibody (given as a gift by David Haake, University of California).

The outer membranes of several serovars, including the nonpathogenic *L. biflexa* serovar Patoc strain Patoc I, grown in the regular medium with added iron were subjected to hemin-agarose binding and analyzed by SDS-PAGE for the expression of the 44-kDa protein.

Nucleotide sequence accession number. The GenBank accession number for the LB191 gene sequence is AE011607.

RESULTS

Cloning and expression of HbpA. The full-length 2,148-bp *hbpA* gene appeared to be toxic to *E. coli*, as was observed by the small colonies and low rate of growth of the transformants. The gene was first cloned into the TA vector and transformed into *E. coli* DH5α. Plasmid isolated from a positive TA clone was cloned into pET28(a) and initially transformed into BL21(DE3). However, as the growth of the transformants was notably slow, the BL21(DE3)/pLysS host was chosen, which showed a relatively higher level of expression of the recombinant protein when grown at 22°C instead of 37°C The 81-kDa rHbpA, purified with an Ni-NTA column, is shown in Fig. 1a (lane 1).

The 1,449-bp fragment, amplified using primers L2 and L3, cloned and expressed in BL21(DE3) yielded a 55-kDa truncated protein ($rHbpA_{55}$) of 483 amino acids lacking 227 amino acid residues from the N terminus. Significant expression of $rHbpA_{55}$ was observed, unlike the case for the full-length HbpA.

Hemin binding by HbpA. We showed the hemin-binding ability of the full-length HbpA by three independent experiments. First, we showed that the Ni-NTA-purified 81-kDa rHbpA bound hemin-agarose beads (Fig. 1a). Further, the recombinant protein could be purified by incubating whole-cell sonicate of the IPTG-induced recombinant *E. coli* with hemin-agarose beads, as seen by the single 81-kDa band upon SDS-PAGE (Fig. 1a). Notably, the 55-kDa $HbpA_{55}$ protein retained the ability to bind hemin (Fig. 1b).

The amount of hemin bound by HbpA was estimated by assaying the inherent heme-dependent peroxidase activity. Figure 2 shows that the amount of bound hemin increased with increasing protein concentration, until at about 3 μg of the protein, the amount of hemin reached a saturation level of about 100 ng of hemin. This provided the second line of evidence for hemin binding by HbpA.

Spectrofluorimetric analysis provided confirmatory evidence for the binding of hemin by HbpA (Fig. 3a). When the pure protein was excited at the excitation wavelength of tryptophan (295 nm), a single emission peak at 388 nm was seen. Upon addition of the ligand (hemin), the intensity of the emitted light decreased, and with increasing ligand addition, a spectral shift to light of shorter wavelength (approximately 370 nm) was noted. The specificity of binding was evident by the dose-dependent spectral changes. The

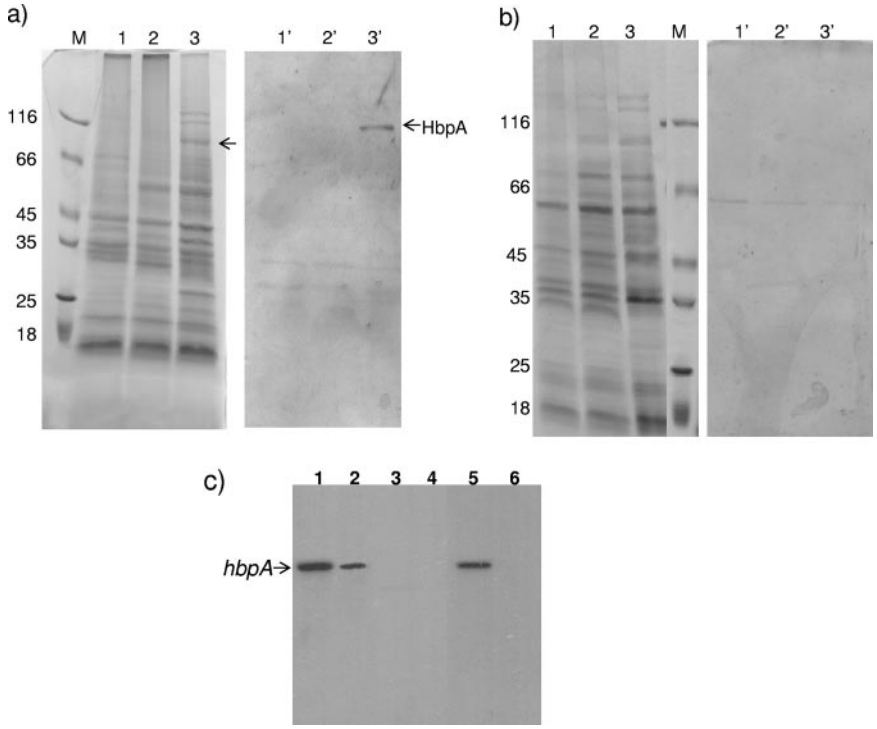

FIG. 4. Expression of HbpA in *L. interrogans* serovar Lai. (a and b) *L. interrogans* serovar Lai strain Lai (a) and *L. borgpetersenii* serovar *Tarassovi* strain Perepelicin (b). Each panel shows the SDS-PAGE profile and the corresponding immunoblot developed with rabbit anti-HbpA antibodies. Lane M, protein marker; lane 1, high-iron cells maintained at 37°C; lanes 2 and 3, low-iron cells maintained at 30°C and 37°C, respectively (see Materials and Methods for a detailed protocol). The lanes in the corresponding immunoblots are represented as 1′, 2′, and 3′, respectively. (c) Southern blot analysis. Chromosomal DNA was digested with HindIII and probed with the 2,148-bp *hbpA* PCR-amplified product. Lanes 1 to 6, *L. interrogans* serovars Lai and Pomona, *L. biflexa* serovar Patoc, *L. borgpetersenii* serovar Tarassovi, *L. interrogans* serovar Autumnalis, and *L. kirschneri* serovar Grippotyphosa, respectively.

association constant of hemin binding was calculated as $6.42 \times 10^4\ M^{-1}$ by using the theoretical fit in the Scatchard plot (Fig. 3b).

HbpA expression is regulated by iron levels. Low-iron conditions were established by the stepwise addition of EDDA to an actively growing culture maintained at 30°C. We could demon-

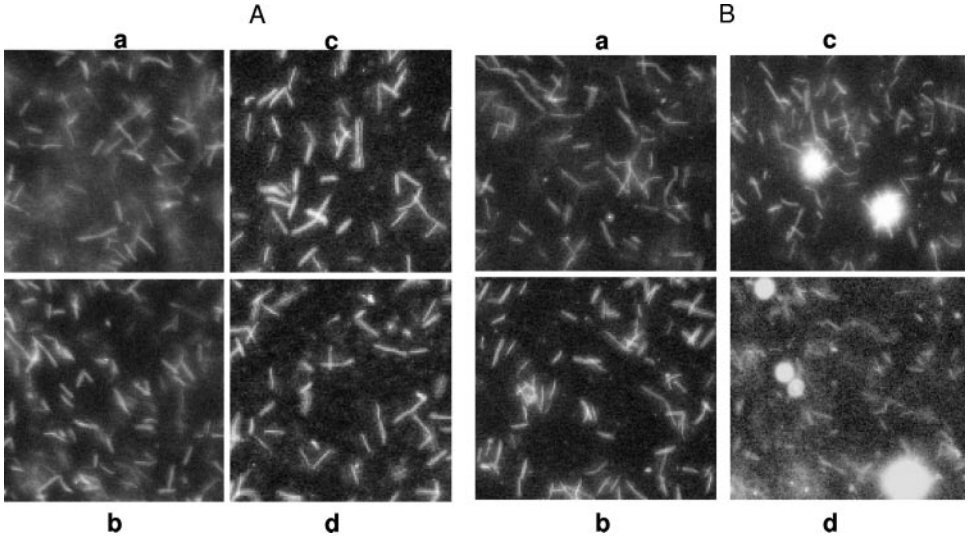

FIG. 5. Agglutination of iron-limited organisms of *L. interrogans* serovar Lai by anti-HbpA antibodies. (A and B) High- and low-iron organisms, respectively. Live organisms (a), live organisms incubated with preimmune serum (b), and live organisms incubated with anti-HbpA antibodies added at dilutions of 1:50 (c) and 1:100 (d) are shown.

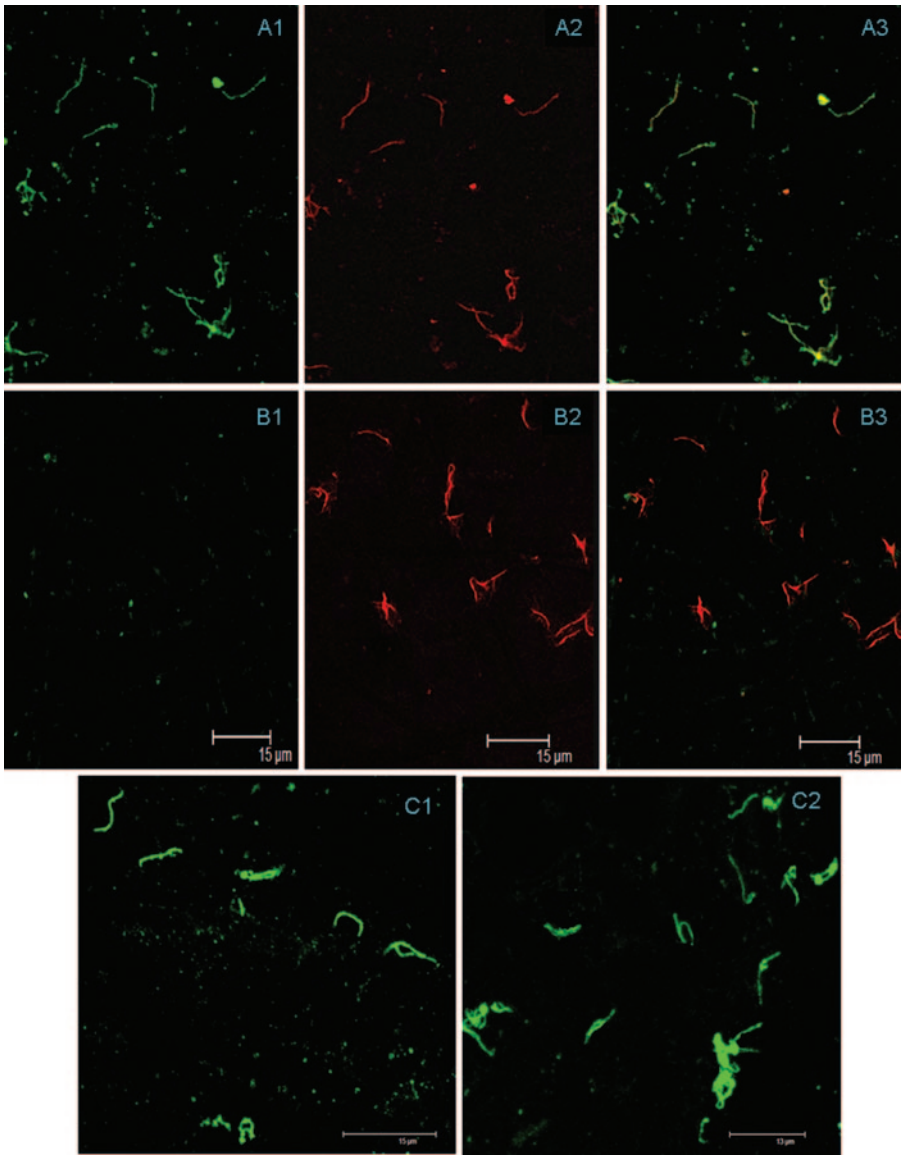

FIG. 6. Demonstration of iron-regulated expression of HbpA in *L. interrogans* by confocal microscopy. Smears of low-iron (A1 to A3) and high-iron (B1 to B3) *L. interrogans* organisms were fixed onto slides, anti-HbpA immunoglobulins were added, and the immunoreactivity was detected using FITC-conjugated secondary antibody. The locations of bacteria were identified by using the DNA counterstain propidium iodide. In both panels A and B, panels 1, 2, and 3 refer to FITC, propidium iodide, and composite images, respectively. The constitutive expression of LipL41 in high-iron (C1) and low-iron (C2) cells from the same batch of organisms, detected using FITC-conjugated secondary antibody, is also shown.

strate the 81-kDa HbpA in low-iron cultures of *L. interrogans* serovar Lai only upon a final incubation of the culture at 37°C for 5 h. The immunoblot shows the single band of reactivity with anti-HbpA antibodies. HbpA was not detected in *L. borgpetersenii* serovar Tarassovi strain Perepelicin (Fig. 4a and b).

Agglutination of leptospires with anti-HbpA antibodies. Low-iron cells of *L. interrogans* serovar Lai showed significant agglutination upon addition of anti-HbpA antibodies. Agglutination was noted within 15 min upon addition of the serum, and after 2 h of incubation, distinct agglutination with lowering of cell numbers was noted. There was no effect of the antibody on high-iron cells. No agglutination was observed in the negative control that included low-iron organisms incubated with preimmune serum from the same animal (Fig. 5).

Immunofluorescence studies with confocal microscopy. Our initial attempts to demonstrate the expression of HbpA in low-iron *L. interrogans* failed when we used whole serum for the primary antibody. Suspecting that agglutination followed by disintegration of the organisms occurred, we subsequently used ammonium sulfate-precipitated immunoglobulins. It is evident that low-iron organisms expressed significant HbpA, in contrast to the almost negligible fluorescence observed with high-iron organisms (Fig. 6). We showed the constitutive expression of LipL41 with the same preparation of organisms.

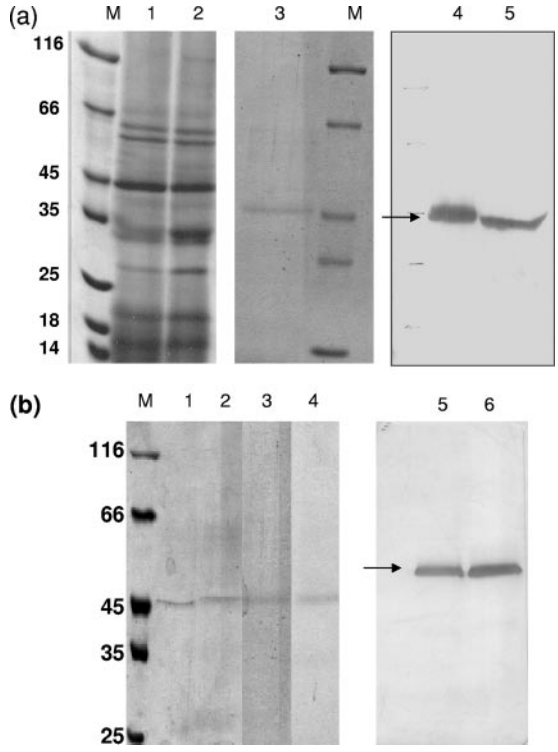

FIG. 7. SDS-PAGE and immunoblotting show that the 44-kDa hemin-binding protein is LipL41. (a) SDS-PAGE and immunoblotting analysis of *L. interrogans* serovar Lai. Lanes developed with Coomassie blue include outer membrane proteins of high-iron (lane 1) and low-iron (lane 2) cells, the 44-kDa protein bound to hemin-agarose preincubated with outer membrane proteins from high-iron cells (lane 3), and molecular weight markers (lane M). Lanes 4 and 5 show the immunoreactivity of anti-LipL41 antibodies with the outer membrane proteins of high-iron cells and hemin-agarose purified 44-kDa protein, respectively. The arrow indicates the 44-kDa band. (b) The 44-kDa proteins from the outer membrane proteins of other serovars. Lanes developed with Coomassie blue include *L. borgpetersenii* serovar Ballum strain MUS 127 (lane 1), *L. biflexa* serovar Patoc strain Patoc I (lane 2), *L. interrogans* serovar Pomona strain Pomona (lane 3), and *L. kirschneri* serovar Grippotyphosa strain Moskva V (lane 4). Lanes 5 and 6 show the immunoreactivity of anti LipL41 with the 44-kDa proteins from *L. interrogans* serovar Lai and *L. biflexa* serovar Patoc, respectively. The arrow indicates the 44-kDa band.

All pathogenic serovars do not elaborate HbpA. We confirmed by Southern blot analysis our earlier observations that HbpA was seen only in some pathogenic serovars (Fig. 4c). A distinct single band was seen in *L. interrogans* serovars Lai, Pomona, and Autumnalis upon hybridization with the full-length *hbpA* probe; the nonpathogenic *L. biflexa* serovar Patoc and the pathogenic *L. borgpetersenii* serovar Tarassovi and *Leptospira kirschneri* serovar Grippotyphosa showed no evidence of the *hbpA* gene in their genomes.

A constitutively expressed 44-kDa hemin-binding protein is identified as LipL41. When outer membranes of both high- and low-iron cells of *L. interrogans* serovar Lai were subjected to hemin-agarose affinity binding, a 44-kDa hemin-binding protein was seen in both of them (Fig. 7). This 44-kDa band was subjected to tryptic digestion, and the sequences of two tryptic peptides were identified as ANLATYYFSTGDFEK and IGNLIGAEAILYIGYQKPYTECSTENK, which identified the protein as LipL41. This observation was confirmed by immunoreactivity of the purified 44-kDa protein with specific anti-LipL41 antibodies (Fig. 7).

Due to the universal expression of LipL41 in pathogenic leptospiral serovars, outer membrane preparations of several serovars, including the nonpathogenic *L. biflexa* serovar Patoc, were subjected to hemin-agarose binding. Unexpectedly, *L. biflexa* also showed the 44-kDa band that reacted specifically with anti-LipL41 antibodies (Fig. 7).

DISCUSSION

Pathogens have evolved diverse systems for acquiring iron, and the ability to utilize heme compounds is particularly important in pathogenic bacteria, as heme is one of the most abundant forms of organic iron in animals (27, 38). Even though the nutritional requirement for iron by pathogenic *Leptospira* is well known (9), little is understood about the mechanisms of iron acquisition by these organisms. Earlier, using a bioinformatics approach, we had reported a TonB-dependent outer membrane protein from the genome of *L. interrogans* serovar Lai (LB191) as a possible hemin receptor (32). In this study, we provide experimental evidence to prove that this 81-kDa protein is a hemin-binding protein, and we term the protein HbpA (*h*emin-*b*inding *p*rotein). The iron-regulated expression and the surface localization of this protein were both demonstrated in this study. In addition, a 44-kDa outer membrane protein, isolated by hemin-agarose affinity binding, is expressed constitutively, independent of iron levels. Sequencing and immunoblotting studies identified this protein as LipL41. Hemin-agarose binding of outer membrane proteins of several serovars, including the nonpathogenic *L. biflexa*, showed a single band of LipL41; this observation was confirmed by immunoblotting.

Some bacterial outer membrane proteins are toxic when overexpressed in *E. coli* (39), with the toxicity being attributed to the 5′ region of the gene that encodes the signal peptide. We found this to be true for the full-length HbpA, with the formation of small colonies and a low level of expression of the 81-kDa rHbpA protein. The truncated $rHbpA_{55}$ protein, bearing the amino residues towards the carboxyl terminus, showed significant expression, however. Three lines of evidence show that HbpA bound hemin. Both rHbpA and $rHbpA_{55}$ bound hemin-agarose effectively, implying that the residues in the N-terminal region are probably not essential for hemin binding. The FRA/PP-NPNL motif associated with hemin binding is seen in HbpA and is retained in the truncated $HbpA_{55}$. Assay of the peroxidase activity of the bound hemin spectrophotometrically and the saturation curve seen in the fluorescence spectrum data show that HbpA bound hemin in a dose-dependent manner. The quenching of the emission spectrum, with maximal intensity at 388 nm, and the spectral shift to a lower wavelength seen in the spectrofluorimetric analysis indicate that hemin bound a specific site on the protein, possibly involving a tryptophan residue.

HbpA probably transports hemin by a mechanism similar to that of other Fe^{3+}-siderophores/hemin receptors (5). As reported earlier (32), it shows protein folding similar to that of the well-characterized ferric siderophore receptors FepA, FecA, and FhuA of *E. coli*. These three *E. coli* receptor pro-

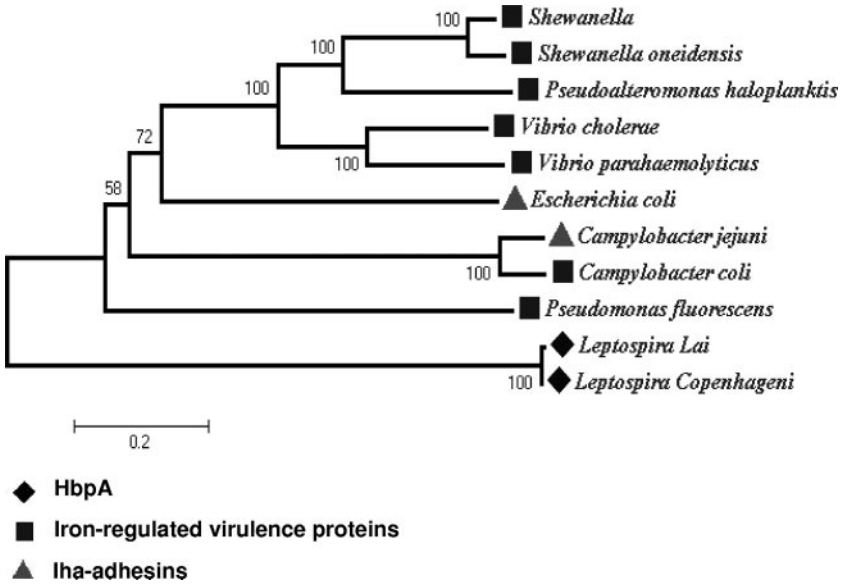

FIG. 8. Phylogenetic analysis of HbpA and other bacterial virulence proteins. The phylogenies generated by neighborhood joining with 400 bootstrap replicates, rooted at midpoint and bootstrap values, are shown as percentages. The numbers refer to the divergence between the sequences.

teins show differences in the sequence of amino acids within the outer loops, thereby accounting for the high affinity and specificity for their respective substrates. However, it is proposed that, by virtue of the similarity in their protein folding, these receptors and several hemin receptors effect internalization of the bound ligand by a common mechanism (5). Upon ligand binding, the receptor interacts with the TonB protein via the TonB box. The energy for this process is derived by the TonB system, consisting of the TonB, ExbB, and ExbD proteins that mediate the transfer of the proton motive force of the cytoplasmic membrane to the outer membrane receptors. The Fe^{3+} complexes are internalized via specific proteins in the periplasm, which deliver the Fe^{3+} and the Fe^{3+} compounds to the ABC transporters in the cytoplasmic membrane. Thus, HbpA is a hemin receptor that functions like other TonB-dependent outer membrane Fe^{3+} transporters, mediating the uptake of Fe^{3+} via hemin. However, further studies are required to understand if the entire molecule is internalized or the Fe^{3+} released at the cell surface is taken up via this protein. Though it has been reported that saprophytic and pathogenic species of *Leptospira* are capable of both de novo synthesis and uptake of heme (12), the specific receptors and mechanism of transport have not been delineated. Sequence alignment and phylogenetic analysis of HbpA with other bacterial proteins show that it shares evolutionary relationships not only with other hemin-binding proteins but also with other bacterial virulence proteins, especially the iron-regulated (Irg) virulence proteins (Fig. 8).

HbpA is expressed on the cell surface of *L. interrogans* when it is maintained under conditions of iron limitation and at 37°C. Growth of pathogenic leptospires under conditions of iron limitation proved to be difficult, as they failed to grow in low-iron media and were sensitive to iron chelators such as 2,2′-dipyridyl. As the optimal temperature for growth of leptospires in vitro is 30°C, we initially facilitated growth of the organisms in regular EMJH medium at 30°C, and after sufficient cell density was reached, the cells were subjected to iron limitation by stepwise lowering of the levels of iron by the addition of increasing amounts of EDDA up to a final concentration of 200 μM. HbpA could not be detected in these cells when they were incubated with hemin-agarose (however, we detected a constitutively expressed 44-kDa hemin-binding protein in both high- and low-iron cells, as discussed below). As pathogenic leptospires grow at 37°C within the mammalian host, we included a final incubation of the live organisms at 37°C for 5 h and showed the temperature-induced expression of HbpA under conditions of iron limitation by immunoblotting with specific anti-HbpA antibodies.

Immunofluorescence studies and agglutination of live leptospires in the presence of anti-HbpA antibodies confirmed the surface expression of HbpA by iron-limited leptospires. Immunofluorescence, observed by confocal microscopy, clearly demonstrated that HbpA was expressed only upon iron limitation. LipL41, a major surface lipoprotein shown to be a hemin-binding protein in this study, served as a control exhibiting constitutive expression under both high- and low-iron conditions. Our initial efforts in demonstrating HbpA expression by immunofluorescence were not successful when we incubated a suspension of live organisms with serum containing anti-HbpA antibodies; subsequently we used ammonium sulfate-precipitated anti-HbpA immunoglobulins instead of whole serum. This was perhaps due to agglutination and disintegration of the organisms in the presence of whole serum. Detailed observations by dark-field microscopy showed that there was significant agglutination of temperature-induced low-iron organisms, with very few intact organisms at 3 h after addition of anti-HbpA antibodies. This is reflected in our observations in the microscopic agglutination test. Notable agglutination was seen in low-iron cells within 15 min of addition of anti-HbpA anti-

bodies, and a significant reduction of organisms was seen by 2 h, with no visible effect in high-iron organisms.

HbpA expression is up regulated by both higher temperature and iron limitation, both conditions likely to be experienced by the invading pathogen. It is known that the mammalian host limits the amount of free iron by a process known as nutritional immunity (17). It is therefore likely that these pathogens encounter conditions of iron deprivation in vivo. Our ongoing studies show the presence of antibodies against HbpA, indicating that HbpA is expressed in vivo. In a recently published list of temperature-regulated proteins (22), HbpA (encoded by LB191) is not present, as it is probably regulated by both temperature and iron. Earlier, Cullen et al. (7), in their analysis of outer membrane proteins under different environmental conditions, could not detect LipL36 and pL50 in leptospires grown under conditions of iron limitation and at temperatures above 30°C. Those authors have indicated that these organisms may not express these proteins during mammalian infection, citing additional evidence (1) that LipL36 is down regulated in vivo. They also report that pL24 was expressed when more free iron was available. Another major outer membrane protein studied in detail by two-dimensional gel electrophoresis by those authors is LipL32, along with its cleavage products. Cleavage of LipL32 occurred when outer membranes were isolated from high-iron organisms and not from low-iron organisms, an observation that could be of biological significance, as LipL32 is thought to play a role in hemolysis (20).

Our earlier study (32) and experimental observations here indicate that not all leptospiral serovars express HbpA and that it appears to be specific for the serovars in the *L. interrogans* species. In the previous study, PCR of the genomic DNAs of several serovars belonging to the *L. interrogans* species showed the *hbpA* gene; it was absent in *L. kirschneri* serovar Grippotyphosa, *L. biflexa* serovar Patoc, and *L. meyeri* serovar Ranarum. In this study, Southern blot analysis of the chromosomal DNA identified the *hbpA* gene in *L. interrogans* serovars Lai, Pomona, and Autumnalis, with no signal seen with DNAs from *L. biflexa* serovar Patoc, *L. borgpetersenii* serovar Tarassovi, and *L. kirschneri* Grippotyphosa. The failure to detect HbpA in *L. borgpetersenii* serovar Tarassovi can be viewed in the light of not finding the corresponding orthologue in the recently sequenced *L. borgpetersenii* genome (6). Preliminary studies in our lab showed the expression of a transferrin-binding protein in serovar Grippotyphosa (unpublished). As it is not uncommon to find several iron uptake mechanisms, further studies are required to understand the different iron uptake systems in *Leptospira*.

In this study, the major emphasis was on characterization of HbpA as a hemin-binding protein. Incidentally, the 44-kDa protein was identified by hemin-agarose affinity binding. This protein, expressed constitutively, proved to be LipL41 by sequencing and immunoblotting with specific anti-LipL41 antibodies. As LipL41 is one of the immunodominant proteins in several leptospiral serovars, we subjected the outer membranes of several leptospiral serovars grown in regular medium to hemin-agarose binding and found that it was expressed in all the serovars tested, including the nonpathogenic *L. biflexa*. The role of LipL41 is not clear. Though it appears to bind hemin, iron levels do not control the expression of this protein. Experimental studies have shown the vaccine potential of this protein, as immunoprotection was seen by the combined use of membrane-purified LipL41 and Omp1 (13). Further understanding of the role of this major surface protein is required to completely assess the diagnostic and vaccine potentials of this protein.

In conclusion, we have demonstrated that HbpA is a hemin-binding protein in *L. interrogans*. The association of this protein, if any, with other reported iron-regulated proteins would be worth further analysis. Of interest is the outer membrane protein LipL32, with its many pI and molecular mass forms and, of greater importance, its role in hemolysis. Therefore, efforts are required not only to unravel the different iron acquisition machineries but also to understand the role of iron in the expression of virulence determinants in the pathogenic leptospires.

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