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Targeted Mutagenesis in Pathogenic *Leptospira* Species: Disruption of the LigB Gene Does Not Affect Virulence in Animal Models of Leptospirosis[▽]

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The pathogenic mechanisms of *Leptospira interrogans*, the causal agent of leptospirosis, remain largely unknown. This is mainly due to the lack of tools for genetically manipulating pathogenic *Leptospira* species. Thus, homologous recombination between introduced DNA and the corresponding chromosomal locus has never been demonstrated for this pathogen. Leptospiral immunoglobulin-like repeat (Lig) proteins were previously identified as putative *Leptospira* virulence factors. In this study, a *ligB* mutant was constructed by allelic exchange in *L. interrogans*; in this mutant a spectinomycin resistance (Spc^r) gene replaced a portion of the *ligB* coding sequence. Gene disruption was confirmed by PCR, immunoblot analysis, and immunofluorescence studies. The *ligB* mutant did not show decrease virulence compared to the wild-type strain in the hamster model of leptospirosis. In addition, inoculation of rats with the *ligB* mutant induced persistent colonization of the kidneys. Finally, LigB was not required to mediate bacterial adherence to cultured cells. Taken together, our data provide the first evidence of site-directed homologous recombination in pathogenic *Leptospira* species. Furthermore, our data suggest that LigB does not play a major role in dissemination of the pathogen in the host and in the development of acute disease manifestations or persistent renal colonization.

Leptospirosis is a widespread zoonosis that has emerged as a major public health problem in developing countries in Southeast Asia and South America (6, 22, 29). This increasingly common disease occurs in poor urban centers subject to frequent flooding (20). Rodents are the main reservoir of the disease, excreting the bacteria in their urine (14, 22). Humans are usually infected through contaminated water. More than 500,000 cases of severe leptospirosis are estimated to occur worldwide each year (46), and the fatality rate is 5 to 20% (29).

The control methods for leptospirosis implemented to date have been ineffective (29). A significant barrier to control and prevention of leptospirosis has been our limited understanding of the pathogenesis of the disease, due in part to the lack of genome sequences and tools to genetically manipulate the pathogens. Most of the barriers have now been overcome. The genomes of two pathogenic species and one saprophytic species have been sequenced (8, 32, 39, 40). Furthermore, we developed a transposon-mediated mutagenesis system for pathogenic *Leptospira* species (7). This advance allowed characterization of the first genetically defined virulence factor in pathogenic *Leptospira* spp. (41). However, the generation of targeted mutants of pathogenic species was not feasible until now.

High-molecular-weight leptospiral immunoglobulin-like repeat (Lig) proteins were previously identified as putative virulence factors in pathogenic *Leptospira* spp. (21, 26, 34). This family of three proteins, LigA, LigB, and LigC, belongs to the superfamily of bacterial immunoglobulin-like (Big) repeat domain proteins, which includes virulence determinants such as intimin from enteropathogenic *Escherichia coli*, invasin from *Yersinia pseudotuberculosis*, and BipA from *Bordetella* spp. (26). This superfamily appears to mediate pathogen-host cell interactions, such as invasion and host cell attachment, during infection. Choy et al. and Lin and Chang recently showed that recombinant Lig proteins can mediate in vitro interactions with host extracellular matrix proteins, including fibronectin, fibrinogen, collagen, and laminin (9, 23). In addition, *lig* genes are upregulated at physiological osmolarity (27) and encode surface-exposed proteins that are strongly recognized by sera from human leptospirosis patients (10, 26, 43). Finally, several studies have shown that Lig proteins are protective antigens in animal models of leptospirosis (21, 35, 42).

In this study, we produced a *ligB* mutant of *L. interrogans* by allelic exchange and evaluated the effect of the deletion in this mutant using both cell adhesion assays and animal models. The results provided the first demonstration of targeted mutagenesis of *Leptospira* pathogenic strains.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Leptospires were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (13, 19) or on 1% agar plates at 30°C and were counted in a Petroff-Hausser counting chamber (Fisher Scientific). *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, a virulent clinical isolate from Brazil (20, 32), was used in all experiments. *E. coli* was grown in Luria-Bertani medium. When necessary, spectinomycin or kanamycin was added to culture media at a concentration of 50 μg/ml.

Polyclonal and monoclonal antibodies. We prepared immune sera against previously described recombinant fragments of LigA (LigANI; amino acid positions 625 to 1225) and LigB (LigBNI; amino acid positions 625 to 1257) (42). These fragments contain the 6th to 13th and 6th to 12th Big repeat domains of LigA and LigB, respectively, and do not include the portions of these molecules which have identical amino acid sequences (26). New Zealand White rabbits were immunized intravenously on days 0, 7, and 14 with three doses of 80 μg of recombinant protein fragments, using aluminum hydroxide as an adjuvant. Rabbits were bled on day 28 to obtain immune sera. For quality control, the reactivities of immune sera with recombinant and native Lig proteins were evaluated using enzyme-linked immunosorbent and immunoblot assays as previously described. Prebleed sera, as well as sera from rabbits immunized with phosphate-buffered saline (PBS) and alhydrogel, were used as control samples. Ascites fluid containing monoclonal antibodies (MAb) against a recombinant LigB protein fragment, LigBrep (26, 42), were provided by José Aleixo, Federal University of Pelotas. The LigBrep fragment corresponds to the six N-terminal Big repeat domains of the LigB molecule (amino acid positions 131 to 649).

Targeted mutagenesis. For allelic exchange, the gene fragment which encodes the LigB nonidentical region (nucleotides 1891 to 5450), also called the B2 region, was amplified from the genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 using primers LigBU1F-SmaI (5′ TCCCCCGGG GCTGAAATTAAAAATACCAGTGGAAG 3′) and P2R-SmaI (5′ TCCCCCG GGCCTATGTAGAGATAAGATCCACTTGC 3′). The PCR product was digested with SmaI and cloned into a PvuII-digested pGKm plasmid vector. The resulting plasmid was then digested with EcoRI, which removed a 699-bp sequence from the *ligB* B2 domain. The Spc^r cassette was amplified from plasmid pGSpc DNA using primers Spc-EcorI3′ (5′ ACGGAATTCAACGTAAAG TAAG 3′) and Spc-EcorI5′ (5′ TCGGAATTCAACGCGTCCCGAGC 3′). The PCR product was digested with EcoRI and subsequently inserted into the plasmid from which the 699-bp *ligB* sequence had been removed to obtain plasmid pB2SK (Fig. 1B). For gene inactivation by plasmid insertion, an internal DNA fragment of *ligB* (nucleotides 1891 to 3771), also called the B1 region, was amplified from the genomic DNA of *L. interrogans* Copenhageni strain Fiocruz L1-130 using primers LigBU1F-SmaI (5′ TCCCCCGGGGCTGAAATTAAAA ATACCAGTGGAAG 3′) and LigBU2R-SmaI (5′ TCCCCCGGGCACTTGG TTTAAGGAATTACAAACT 3′). The PCR product was digested with SmaI and cloned into a PvuII-digested pGSpc plasmid vector, resulting in plasmid pB1S. Plasmids pGKm and pGSpc (complete sequences are available on request) were derived from the cloning vector pGEM-7Zf(−) (Promega Corporation, Madison, WI) as previously described (15). We used the *Enterococcus faecalis* kanamycin and *Staphylococcus aureus* Spc^r cassettes as previously described (5).

The plasmid constructs, which are not replicative in *Leptospira* spp., were used to deliver the inactivated allele into *L. interrogans* by electroporation. Cells were grown to exponential phase (optical density at 420 nm, 0.10 to 0.20) and then centrifuged at 4,000 × *g* and concentrated to obtain 10^{10} bacteria/ml in sterile water. Suicide plasmids containing the inactivated allele were subjected to 5 to 30 s of UV treatment (254 nm, 400 μW/cm^2) using a UV chamber (GS Gene linker; Bio-Rad). Two hundred microliters of cells was electroporated (1.8-kV, 200-Ω, 25-μF electric pulse in a prechilled 0.2-cm-diameter cuvette) in the presence of 100 to 500 ng of plasmid DNA and then transferred to 1 ml of EMJH liquid medium, in which the cells were incubated for 24 h at 30°C. The bacteria were then plated on EMJH medium supplemented with spectinomycin (50 μg/ml). After 4 to 6 weeks of incubation, Spc^r colonies were picked and examined for allelic exchange in the target gene by PCR, Western blotting, and immunofluorescence analysis.

Genomic DNA analysis. Genomic DNA was prepared from liquid cultures by use of a cell DNA purification kit (Maxwell, Promega, Madison, WI). To check for double homologous recombination, primers B2EF (5′ CATACACACTTGT AGTCAACAAACAAG 3′) and B2ER (5′ CGTAACGTAATTCGGAACCG 3′) and primers LBN (5′ GGGAATTCCATATGAAGAAAATATTTTGTAT TTCG 3′) and P2R (5′ TATGTAGAGATAAGATCCACTTGC 3′) were used for amplification of the *ligB* locus (Fig. 1A). Gene inactivation by plasmid insertion was confirmed by using primers B1F (5′ ACCTGGAATTCCTCTAA TACGGATATT 3′) and B1R (5′ GAATATAAAGGTTTGGAAAAAGA AACG 3′) for PCR amplification.

Immunoblotting. Mutant and wild-type *L. interrogans* Fiocruz L1-130 strains were grown in EMJH medium until the optical density at 420 nm was 0.2. *Leptospira biflexa* was also used as a control in these experiments. Bacteria were washed in PBS. After the concentration was adjusted to 2×10^8 bacteria/per well (20 μl), the cells were solubilized in 62.5 mM Tris hydrochloride (pH 6.8)–10% glycerol–5% 2-mercaptoethanol–2% sodium dodecyl sulfate. Crude protein extracts were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a discontinuous buffer system. After transfer to nitrocellulose membranes, immunoblots were blocked in 0.05 M Tris-buffered saline (pH 7.4)–0.05% (vol/vol) Tween 20 with 5% (wt/vol) nonfat dry milk. The blots were washed, incubated for 1 h at room temperature with a 1,000-fold dilution of mouse ascites containing MAb to the LigB identical repeat region (LigA/B) or with a 10,000-fold dilution of hyperimmune rabbit antisera to LipL41, and probed with goat anti-mouse and anti-rabbit immunoglobulin G (IgG) antibodies conjugated to alkaline phosphatase (Sigma). Immunoblots were developed in a nitroblue tetrazolium—5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Bio-Rad).

Immunofluorescence assays. Immunofluorescence labeling was performed using a modified protocol of Cullen et al. (12). Suspensions containing 10^7 live leptospires in 10 μl of PBS were placed on poly-L-lysine-coated (Sigma) slides and incubated for 1 h in a humidified chamber. The slides were washed twice with PBS, blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma) (PBS-BSA), and incubated for 1 h with hyperimmune rabbit antisera to the LigB nonidentical region (LigBNI) and the LigA nonidentical region (LigANI) (diluted 1:100 in PBS-BSA) and with control rabbit antisera to a leishmanial antigen. The slides were washed gently with PBS-BSA and incubated with donkey anti-mouse IgG antibodies conjugated to Alexa dye (Molecular Probes) or with goat anti-rat IgG antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. The slides were washed twice with PBS-BSA and incubated with 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 1 h at room temperature. The slides were washed and then mounted in antifading solution (Prolong; Molecular Probes) and visualized by fluorescence microscopy (Olympus BX51).

Hamster model of acute infection. Groups of four Golden Syrian male hamsters that were 5 to 8 weeks old were inoculated intraperitoneally with 10, 10^2, 10^4, and 10^6 cells of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains. Negative control animals were inoculated intraperitoneally with 1 ml of EMJH medium. Animals were monitored daily for clinical signs of leptospirosis (prostration and jaundice) and survival. Surviving animals were killed after a 21-day postchallenge follow-up period. The 50% lethal dose (LD_{50}) of *L. interrogans* strain Fiocruz L1-130 in 5- to 8-week-old hamsters was approximately 10^1 leptospires. Culture isolation and immunofluorescence studies were performed using kidney and liver samples (42) to determine whether surviving animals had a persistent infection. The protocols used for animal experiments followed the guidelines of the Animal Care and Use Committee of Fundação Oswaldo Cruz.

Rat model of chronic infection. Groups of four or eight Wistar rats (Fiocruz, Rio de Janeiro, Brazil) that were 4 to 5 weeks old were inoculated intraperitoneally with 10^8 cells of the wild-type and *ligB* mutant strains in 1 ml EMJH medium. Control animals were inoculated intraperitoneally with 1 ml of sterile EMJH medium. Animals were sacrificed 15 days after infection. Necropsies were performed immediately after sacrifice. Kidney and liver samples were fixed in 4% formalin, embedded in paraffin, and cut into 4- to 5-μm sections for conventional histology analysis. Renal tissue samples were homogenized in 5 ml of EMJH liquid medium for 10 min. After separation of the supernatant from the tissues, 500 μl of the supernatant was used to inoculate 5 ml EMJH liquid medium, which was subsequently incubated at 29°C. The cultures were examined weekly for growth by dark-field microscopy for up to 6 weeks.

Histopathology studies. Groups of three hamsters were inoculated with 10^6 cells of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains and euthanized on day 9 postchallenge. Tissues (liver, kidneys, and lungs) were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned using routine histological procedures to obtain 4- to 5-μm sections that were then stained with hematoxylin and eosin. For immunohistochemistry analysis, the paraffin was removed from the sections with xylene and ethanol. The tissues were blocked by incubation of sections with 1.0% BSA at room temperature for 20 min. The tissues were incubated with a 1,000-fold dilution of antiserum to LipL32 (17) at room temperature for 1 h. Samples were treated with 0.3% hydrogen peroxide for 15 min at room temperature and then incubated at room temperature for 30 min with goat anti-mouse or anti-rabbit antibodies conjugated to peroxidase (Histostain-Plus kit; Invitrogen). Enzyme reactions were developed using 3,3′-diaminobenzidine (Sigma).

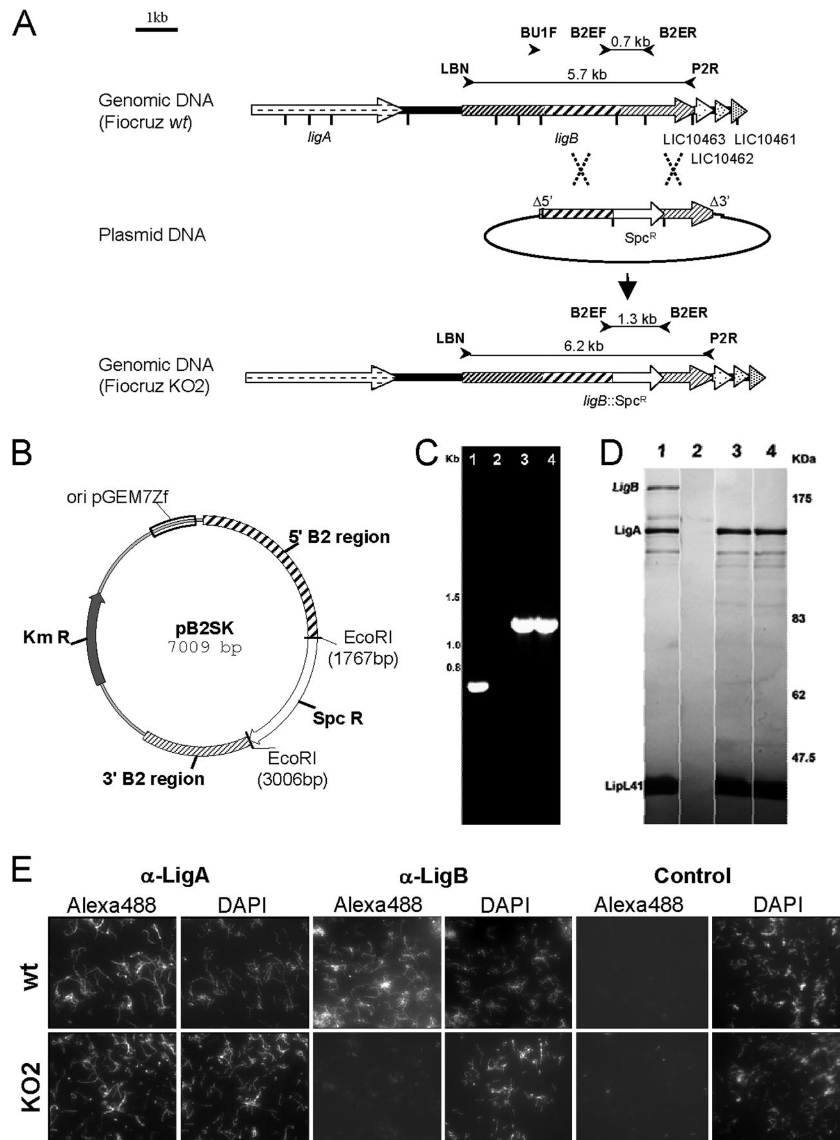

FIG. 1. Disruption of *ligB* in *L. interrogans* strain Fiocruz L1-130. (A) Schematic representation of the genotype of the parental (Fiocruz *wt*) and *ligB* (Fiocruz KO2) mutant strains. The LigB protein has a tripartite structure which includes an N-terminal identical repeat region, a nonidentical repeat region, and the C-terminal region. The vertical bars indicate EcoRI restriction sites. The locations of primers used to check for allelic exchange, as well as the expected sizes of amplified products, are indicated. (B) Map of the pB2SK plasmid, in which the Spc^r cassette was inserted between EcoRI sites in the B2 region of *ligB*. (C) PCR amplification of chromosomal DNA from the *L. interrogans* wild-type strain (lane 1), *L. biflexa* strain Patoc1 (lane 2), the *L. interrogans ligB* KO2 mutant (lane 3), and the *L. interrogans ligB* KO2 mutant reisolated from hamsters after infection (lane 4) with primers B2EF and B2ER, as shown in panel A. (D) Western blot of LigA and LigB expression in the *L. interrogans* wild-type strain (lane 1), *L. biflexa* strain Patoc1 (lane 2), the *L. interrogans ligB* mutant (lane 3), and the *L. interrogans ligB* mutant which was reisolated from hamsters (lane 4). Blots were also probed with LipL41 antiserum as a reference. (E) Immunofluorescence assays were performed with *L. interrogans* wild-type (wt) and *ligB* mutant (KO2) strains. Strains were labeled with antibodies against LigANI (α-LigA0), LigBNI (α-LigB), and a control. Alexa- and fluorescein isothiocyanate-conjugated secondary antibodies were used to detect surface-bound antibodies to LigANI and LigBNI, respectively. A DAPI counterstain was used to document the presence of leptospires. The photomicrograph show the results of one of three representative experiments.

Host cell adhesion assay. Madin-Darby canine kidney (MDCK) cells were harvested by treating cell cultures with 0.05% trypsin and 0.02% EDTA in PBS and then plated on 24-well plates in Dulbecco's modified Eagle's medium (Cultilab) without antibiotics. Cell viability was determined by trypan blue exclusion, and 500-μl portions of cell suspensions containing 2×10^5 cells per ml were layered on round glass coverslips in 24-well tissue culture plates. The plates were incubated for 24 h and washed twice with PBS to remove nonadherent cells. Bacteria were suspended in warm (37°C) cell culture medium at a concentration of 2×10^7 cells per ml. Wild-type and *ligB* mutant strains were used at the same time in each experiment. A 500-μl aliquot of each bacterial suspension was then added to the wells at a bacterium/cell ratio of 100:1. The plates were incubated under static conditions for 1 h at 37°C (30). Experiments were performed in triplicate. Coverslips were washed three times in PBS to remove nonadherent bacteria. An immunofluorescence analysis was performed as described above. The first antibody was anti-LipL32 MAb or anti-Omp Salmonella MAb, and the second antibody was anti-mouse antibody conjugated with Alexa 488 (Molecular Probes). DAPI and Alcian Blue were used to stain the nucleus and the cytoplasm, respectively. The numbers of leptospires and MDCK cells were deter-

mined by examining 10 high-power fields during fluorescence microscopy. Student's *t* test was used to evaluate the significance of differences between the numbers of associated leptospires per host cell in incubations with wild-type and *ligB* mutant strains.

RESULTS

Allelic exchange mutagenesis of *L. interrogans ligB*. Pathogenic *Leptospira* species possess between one and three *lig* genes. *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 contains two *lig* genes, *ligA* (3,675 bp) and *ligB* (5,673 bp), which encode polypeptides with molecular masses of 128 and 201 kDa, respectively. The third gene, *ligC*, was identified as a pseudogene in this *L. interrogans* strain (26). The *ligB* locus was used as a target for mutagenesis by allelic exchange in *L. interrogans* strain Fiocruz L1-130. A gene replacement construct was generated by cloning *ligB* into a suicide vector, which deleted a portion of the *ligB* open reading frame, which was replaced by an Spc^r cassette. The amounts of homologous *L. interrogans* DNA present on the two sides of the spectinomycin marker were 1.8 and 1 kb (Fig. 1A).

The origin of replication used in the plasmid construct was that from pGEM7Zf, which is nonfunctional in *Leptospira* spp. Thus, any Spc^r colonies arising after electroporation of this plasmid into *L. interrogans* should have resulted from recombination of the plasmid with the host genome. Strain Fiocruz L1-130 was electroporated with the UV-irradiated plasmid construct as previously described (38) and plated on solid medium containing spectinomycin. A total of six transformation experiments were performed, one of which yielded two transformants. The two mutant clones were obtained on the same plate and may have been mutants of siblings.

To confirm that homologous recombination events occurred at the origin of the Spc^r phenotype, the *ligB* locus was analyzed by PCR mapping of genomic DNA obtained from *L. interrogans* transformants. PCR amplification with primers B2EF and B2ER, which normally produce a 0.7-kb product with wild-type *L. interrogans*, generated a 1.3-kb DNA fragment with the two Spc^r recombinants analyzed (Fig. 1B). The amplified product was the expected size if a *ligB* mutant resulted from integration of the Spc^r cassette by double-crossover recombination in the *ligB* chromosomal locus (Fig. 1A and B). Furthermore, PCR amplification with primers B2N and P2R yielded a product which had a size that was consistent with a gene replacement event in *ligB* loci. Immunoblotting with an anti-LigA/B MAb confirmed that LigB-reactive polypeptides were not present in the mutant (Fig. 1C). Moreover, by evaluating the reactivity with antibodies raised against LigA, we were able to detect LigA, which is encoded by the *lig* gene located upstream of *ligB* (26). This finding indicates that *ligB* inactivation does not modify *ligA* expression. Immunofluorescence studies also demonstrated that the *ligB* mutant did not express LigB, whereas the wild-type strain did express this protein (Fig. 1D). In contrast, antisera to LigA labeled live *ligB* mutant and wild-type leptospiral strains similarly.

By using another approach, we cloned an internal fragment of *ligB* lacking the 5′ and 3′ ends of the open reading frame in a suicide vector. Five transformants were recovered after electroporation in *L. interrogans* strain Fiocruz L1-130 in one of four transformation experiments. With all clones tested we obtained integration of the plasmid via a single crossover event, which generated two copies of the targeted gene, one with a deletion at the 5′ end of the gene and the other with a deletion at the 3′ end, thereby rendering it inactive, as confirmed by immunoblotting (data not shown).

TABLE 1. Virulence of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains in the hamster model of leptospirosis

Strain	No. deaths (% of total) with a challenge dose of[a]:			
	10^6 Bacteria	10^4 Bacteria	10^2 Bacteria	10^1 Bacteria
Expt 1				
Wild type	4 (100)	4 (100)	4 (100)	ND[b]
ligB KO2 mutant	4 (100)	4 (100)	4 (100)	ND
Expt 2				
Wild type	4 (100)	4 (100)	4 (100)	ND
ligB KO2 mutant	4 (100)	4 (100)	4 (100)	ND
Expt 3				
Wild type	ND	3 (75)	4 (100)	3 (75)
ligB KO2 mutant	ND	4 (100)	4 (100)	4 (100)

[a] Groups of four hamsters were inoculated with each challenge dose.
[b] ND, not determined.

In addition to the differences in genotype mentioned above, the transformants resulting from allelic exchange and plasmid insertion did not produce LigB; hence, the term *ligB* mutant refers to the double-crossover recombinant KO2 mutant below unless indicated otherwise.

Loss of the *ligB* gene does not affect virulence and persistence in animal models. The *ligB* mutant and wild-type strains had similar cell growth kinetics in liquid EMJH medium (the generation time for the parent and mutant strains was approximately 20 h). Inactivation of *ligB* did not affect cell morphology and motility.

In order to determine whether LigB may have a role in virulence in vivo, we evaluated *ligB* mutants and the parental wild-type strain using the standard hamster model for acute leptospirosis. Different numbers of organisms (log increases in the challenge dose) were inoculated intraperitoneally to produce infection. The proportion of hamsters which died and the proportion which survived for each bacterial concentration were used to calculate the LD_{50}. Three independent experiments in which groups of four animals were infected with each challenge dose were performed (Table 1). The LD_{50} was less than 100 bacteria for both the wild-type and mutant strains. Thus, the lack of LigB expression did not result in loss of virulence as measured by the LD_{50}. In addition, no significant differences in the time to death were observed with the *ligB* and wild-type strains (data not shown). The general health status of the hamsters infected with the *ligB* and wild-type strains was also assessed. Infections with the *ligB* and wild-type strains produced similar pathological findings (jaundice, pulmonary hemorrhage, dissociation of hepatic trabecula, and acute damage of renal tubular epithelia with cell swelling in proximal segments). The immunohistochemistry results showed the same distribution of *Leptospira* in the renal parenchyma (Fig. 2).

The virulence of the *ligB* mutant was evaluated using the rat model for renal colonization. In three separate experiments, groups of four or eight rats were infected intraperi-

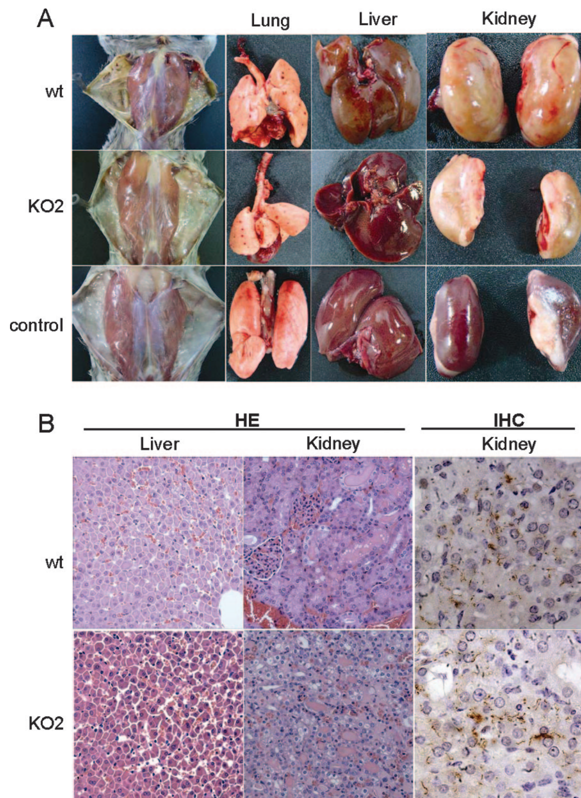

FIG. 2. Pathology in hamsters infected with the *ligB* mutant. (A) Gross appearance of hamsters infected with the wild-type (wt) and mutant *ligB* mutant (KO2) strains and a representative uninfected control hamster. (B) Livers and kidneys from hamsters infected with the wild-type and *ligB* mutant strains of *L. interrogans*. Tissues were stained with hematoxylin and eosin (HE) (magnification, ×400), and the immunohistochemistry analysis was performed with antiserum specific for LipL32 (IHC) (magnification, ×1,000).

toneally with 10^8 cells of either the *ligB* mutant or wild-type strain as previously described (1). Rats were sacrificed 15 days after infection. The *ligB* mutant behaved like the wild type. High levels of both strains were recovered in rat kidneys (Table 2). In hamster and rat experiments, the double-crossover disruptant KO2 was recovered from animals at the time of sacrifice and 2 weeks postchallenge, respectively, and the genotype was confirmed by PCR and immunoblot analysis (Fig. 1). This suggests that the *ligB* disruption was stable in the absence of selection.

In vitro adherence of the *ligB* mutant to MDCK cells. Interactions of *L. interrogans* wild-type and mutant strains with cultured epithelial cells were assayed by examining the adherence of leptospires to epithelial monolayers of MDCK cells. Cell monolayers were incubated using a multiplicity of infection of 100 bacteria per MDCK cell, and subsequent binding was quantified by microscopic analysis. There was not any statistically significant difference between the *L. interrogans ligB* mutant and the wild-type strain in the number of bacteria associated with MDCK cells (Fig. 3). These findings suggest that the *ligB* genotype does not influence in vitro host cell association.

DISCUSSION

Compared to other bacterial species, the genetic data for leptospires and determination of the molecular basis of the pathogenesis of these organisms are in their infancy. Analysis of the complete genome sequences of pathogenic *Leptospira* species revealed that more than 50% of the predicted open reading frames did not exhibit similarity to genes encoding proteins with known functions (8, 32, 39, 40). Until a method for constructing pathogenic leptospire mutants is developed, any function of leptospire proteins, including virulence factors, remains speculative. Previous attempts to inactivate genes in pathogenic *Leptospira* species have been unsuccessful. The putative role of LigB in virulence (21, 26, 34) prompted us to generate an *L. interrogans ligB* mutant.

We used approaches used previously for saprophytic *Leptospira* species (5, 16, 24, 25, 28, 37, 38, 45) to carry out gene targeting by homologous recombination in the pathogen *L. interrogans*. Although the efficiency of the transformations was low, our results show the feasibility of performing allelic exchange in pathogenic *Leptospira* spp. by homologous recombination. Our previous attempts to generate homologous recombination in *L. interrogans* were not successful (unpublished data), presumably due to the target gene chosen. The use of a large region of homologous DNA (more than 1 kb) may have increased the probability of homologous recombination. The *ligAB* locus appears to be the target of fragment rearrangements and recombination events. It has been suggested that *ligA* was created from *ligB* by gene duplication, since the fragments which encode the first six Big domains are identical in the two genes (26). Furthermore, sequence analysis of *lig* genes from collections of pathogenic *Leptospira* species resulted in evidence of recombination between *Leptospira* species at this locus (unpublished data). Finally, in addition to allelic exchange derived from a double homologous recombination event, we showed that targeted integration of a suicide plasmid which contains a 5′- and 3′-truncated fragment of the gene of interest can facilitate targeted mutagenesis in *L. interrogans*.

For members of the superfamily containing the Big proteins, previous studies have demonstrated that compared to the wild-

TABLE 2. Renal colonization of *Rattus norvegicus* with the wild-type and *ligB* mutant strains of *L. interrogans* Fiocruz L1-130 after experimental challenge

Strain	No. animals with evidence of *Leptospira* renal colonization (% of total) based on[a]:	
	Culture isolation	Immunofluorescence
Expt 1		
Wild type	6 (75)	8 (100)
ligB KO2 mutant	8 (100)	8 (100)
Expt 2		
Wild type	4 (100)	4 (100)
ligB KO2 mutant	4 (100)	4 (100)
Expt 3		
Wild type	8 (100)	8 (100)
ligB KO2 mutant	5 (63)	5 (63)

[a] Groups of eight rats and groups of four rats were inoculated with 10^8 leptospires in experiments 1 and 3 and in experiment 2, respectively.

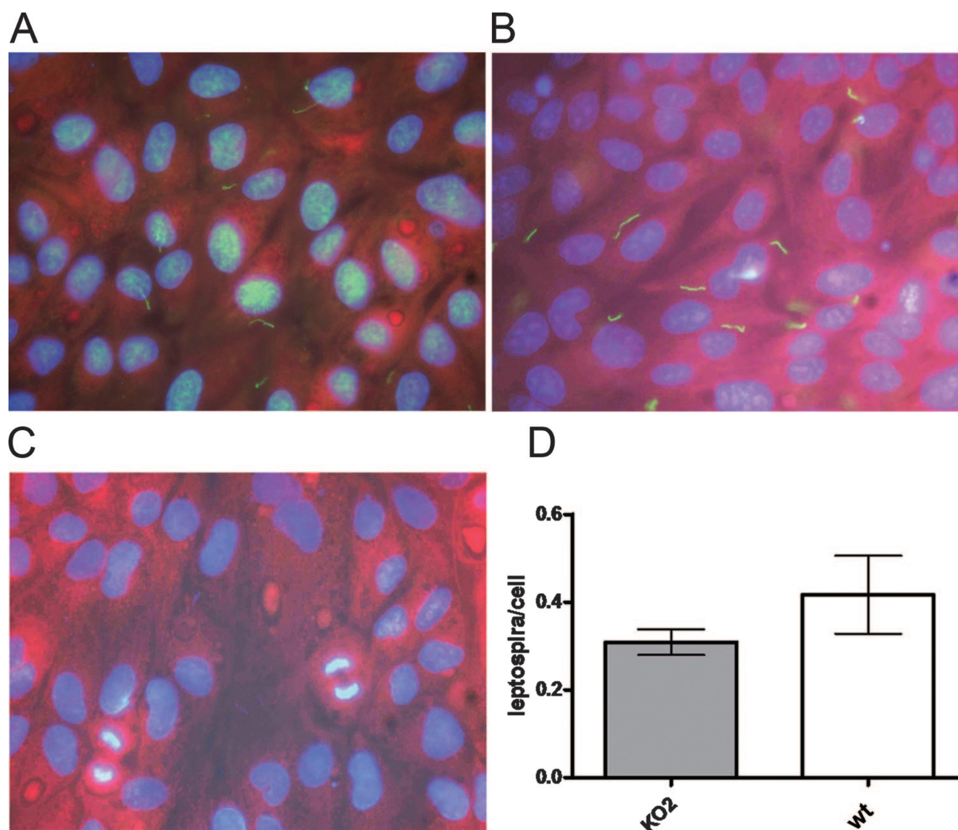

FIG. 3. Adherence of the *L. interrogans ligB* mutant to MDCK epithelial cells. The adherence of the *L. interrogans* wild-type (A) and *ligB* mutant KO2 (B) strains to MDCK epithelial cells was examined. Anti-*Salmonella* OmpA MAb was used as a control (C). Representative micrographs obtained by fluorescence microscopy are shown. (D) Attachment ratios (means ± standard deviations) determined using 10 random fields. wt, wild type.

type strain, an intimin-deficient enteropathogenic *E. coli* strain is defective for adherence to cultured cells and for intestinal colonization (33). Similarly, analysis of a *Y. enterocolitica inv* mutant suggested that invasin is necessary for efficient translocation of the bacteria across the intestinal epithelium (36). *ligB* is conserved among all pathogenic *Leptospira* species and is upregulated when bacteria confront the host environment (26, 34). Furthermore, expression of *ligB* is correlated with the virulence status of *Leptospira* strains (26). Therefore, the working hypothesis has been that LigB is essential for the bacteria to survive, disseminate, and/or colonize in the host.

Yet we found that loss of *ligB* was not associated with a loss of virulence phenotypes. Inoculation of *ligB* mutants produced the same acute disease manifestations and lethal outcomes that were observed in hamsters infected with wild-type strains. In addition, inoculation of *ligB* mutants resulted in efficient renal colonization in experimental rats similar to that observed with wild-type strains.

Moreover, we found that the *L. interrogans ligB* mutant was able to adhere to epithelial cells in vitro. The interaction of *L. interrogans* with host cells is critical for dissemination in the host (4). Although the LigB protein has been shown to bind in vitro to host extracellular matrix moieties (9, 23), our findings suggest that there may be other modes of leptospiral attachment to host epithelial cells. As a caveat, we did not examine whether *ligB* mutants bind to extracellular matrix components, including fibronectin, and further studies are required to evaluate this possibility. Furthermore, the mechanism of association of the pathogen with the host cell in vivo may be quite different than what is observed in vitro; therefore, we cannot exclude the possibility that Lig proteins mediate host cell interactions based on observations made with in vitro assays alone.

Because of the location of the Spc^r cassette in the 3′ end of *ligB*, a truncated LigB protein could have been expressed. However, immunoblot analysis using polyclonal and monoclonal antibodies against recombinant fragments located upstream of the disruption site did not allow identification of any fragments in the mutant strains. Furthermore, whereas these antibodies stained strongly with the wild-type strain in immunofluorescence studies, no signal was associated with the mutant strains. It is therefore unlikely that a truncated *ligB* fragment was expressed in the mutants.

In the present study, the data obtained with the *ligB* mutant suggest that an absence of LigB does not lead to a loss of virulence and a loss of colonization in the acutely and chronically infected animal models, respectively. In our challenge experiments, hamsters and rats were infected by intraperitoneal inoculation of leptospires. We cannot exclude the possibility that LigB may play a role in penetration of the host or other early events during infection. Alternatively, the fact that the *ligB* mutant remained virulent may have been due to func-

tional redundancy in the bacteria. The numerous lipoproteins which are present in leptospires (11) in addition to the LigB protein may compensate for the loss of LigB expression. Several surface-associated *Leptospira* proteins, including LigA, have been shown to interact in vitro with extracellular matrix components (2, 3, 18, 31, 44). Thus, the function of LigB may be replaced to various extents by other lipoproteins which may play a role in host-cell interactions. LigA and LigB proteins contain Big domains that may have redundant functions (9, 27, 34). Choy et al. demonstrated that domains within LigA and LigB proteins bind specifically to fibronectin in vitro (9). A phenotype distinct from that of the parental strain may occur only when both genes are disrupted. Therefore, further studies should include generation of *ligA* and *ligAB* mutants.

In conclusion, we demonstrated for the first time that site-directed homologous recombination can be successfully achieved in pathogenic *Leptospira*. The approaches used in this study, therefore, make it feasible to produce knockout mutations in putative virulence-associated genes in *Leptospira* and evaluate the roles that these genes may play in leptospiral pathogenesis.

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