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Catecholamine-Modulated Novel Surface-Exposed Adhesin LIC20035 of *Leptospira* spp. Binds Host Extracellular Matrix Components and Is Recognized by the Host during Infection

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ABSTRACT In this study, the effect of the host stress hormone catecholamine on *Leptospira* gene transcripts encoding outer membrane proteins was investigated. There was no impact of catecholamine supplementation on the *in vitro* growth pattern of *Leptospira interrogans*; however, 7 genes out of 41 were differentially transcribed, and the effect was reversed to the basal level in the presence of the antagonist propranolol. Comprehensive analysis of one of the differentially regulated proteins, LIC20035 (in serovar Copenhageni)/LB047 (in serovar Lai) (due to catecholamine supplementation), revealed immunogenicity and ability to adhere to host extracellular matrices. Protease accessibility assay and phase partition of integral membrane proteins of *Leptospira* showed LIC20035/LB047 to be an outer membrane surface-exposed protein. The recombinant LIC20035 protein can be serologically detected using human/bovine sera positive for leptospirosis. Moreover, the recombinant LIC20035 can bind to diverse host extracellular matrices, with a higher affinity toward collagen and chondroitin sulfate.

IMPORTANCE Leptospirosis is a neglected tropical disease of global importance. This study aimed to identify outer membrane proteins of pathogenic *Leptospira* responding to host chemical signals like catecholamines, with the potential to serve as virulence factors, new serodiagnostic antigens, and vaccine candidates. This study mimicked the plausible means by which *Leptospira* during infection and hormonal stress intercepts host catecholamines to disseminate in host tissues.

KEYWORDS *Leptospira*, catecholamines, qRT-PCR, differential transcription, stress, LB047, LIC20035, stress hormone

Leptospira organisms are classified as spirochetes of pathogenic and nonpathogenic forms with a variety of different habitats in nature (1). However, the pathogenic *Leptospira* is maintained in a complex enzootic cycle involving multiple host species and the variable environmental niche. The host specificity of bacterial pathogens is determined by interactions between the pathogens and their host factors (2). Host stress hormones are among these host factors which lead to adaptation of various pathogenic microbes in the human or animal body upon infection (3–6). The primary hormones synthesized under stress conditions in animals and humans are the catecholamines. These are a large group of amine hormones derived from tyrosine and include primarily epinephrine (Epi) and norepinephrine (NE). Catecholamines are identified as the sympathetic neuroendocrine mediators of the fight-or-flight (acute stress) response of the host (7). The pathogenic and nonpathogenic microbes intercept host catecholamine and use it as an environmental cue to alter its growth and virulence (7). In the last few years, numerous studies have been done in regard to the effect of

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catecholamines on the differential expression of outer membrane proteins (OMPs) of infectious agents. In *Escherichia coli*, catecholamines can induce genes associated with adhesion to host cells (4, 8–10), affect chemotaxis, affect colonization of HeLa cells (3), and can even enhance toxin production in them (6, 11). In the same way, in response to catecholamine, *Actinobacillus pleuropneumoniae* (12) and *Borrelia burgdorferi* (13) actively regulate virulence genes. Such studies have proved that the pathogens take advantage of the host response upon infection and illustrate the way in which pathogens can intercept host hormonal signals to their advantage. Substantial evidence from aforementioned studies conveys that bacteria have a catecholamine response system(s) that possesses pharmacological similarity to the mammalian adrenoceptors and dopamine receptors.

Outer membrane proteins of *Leptospira* are core components by which pathogenic *Leptospira* organisms interact with the host and play an essential role as adhesins (14), receptors for various host molecules (15), and key mediators for adaptation to change in the environment (16). There are three classes of outer membrane protein of *Leptospira* identified to date, *viz*., outer membrane lipoprotein, transmembrane protein, and the peripheral membrane protein (17). Several leptospiral OMPs have been reported to have the capacity to adhere to extracellular matrix (ECM) components of the host and is an essential requisite for tissue invasion.

Taking into consideration the above-listed points, this study aimed to understand modulation of OMPs gene transcription in *Leptospira interrogans* on exposure to catecholamines under *in vitro* conditions. We report transcript analyses using the real-time reverse transcription-PCR (qRT-PCR) technique of selective genes encoding OMP of serovars Lai and Copenhageni in response to Epi/NE and their antagonist propranolol (PO). It is anticipated that this approach will facilitate the identification of OMPs responding to host chemical signals with the potential to serve as virulence factors, new serodiagnostic antigens, and vaccine candidates. As an initial step toward the comprehensive understanding of the effect of catecholamines on the transcription of membrane proteins of *L. interrogans*, one out of seven genes, *viz*., *LIC20035* (in serovar Copenhageni)/*LB047* (in serovar Lai), showing a response to catecholamines was further characterized. The coding sequence (CDS) *LIC20035/LB047* encodes a hypothetical membrane protein (50 kDa) of pathogenic *Leptospira* and was found to be repressed in the presence of Epi, the effect of which was blocked in the presence of its antagonist. Additionally, recombinant LIC20035 (r-LIC20035) shows a higher affinity for host extracellular matrices like collagen and chondroitin sulfate.

RESULTS

Effect of catecholamine supplementation on the growth of *Leptospira*. Human adrenergic agonists can affect growth of several medically important bacteria due to their ability to act as siderophores providing elemental iron essential for metabolic activity (18). A growth rate analysis of *L. interrogans* serovar Lai was performed in the presence and absence of Epi/NE (500 μM) and the antagonist PO (500 μM) to examine any effect on the growth of spirochetes in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium under *in vitro* culture conditions (IVCL) at 29°C. There was no statistical difference in the growth rate of the spirochetes at any time point (0 to 120 h) in the presence of catecholamines or the antagonist (Fig. 1). These results show that the working concentration of catecholamines (500 μM) used for the experiments was not affecting the growth of the bacterium under IVCL. Since the catecholamines have also been shown to be involved in modulating bacterial virulence (12, 13), it evoked interest to study their effects on the selective transcriptome of the spirochetes.

Effect of catecholamines on spirochete selective gene transcripts. A total of 41 genes of *L. interrogans* serovar Lai, which were predicted using the program PSORT to encode membrane proteins, were chosen. A few genes with assigned function, e.g., those involved in an iron uptake, were also included for analyzing the *in vitro* transcription pattern in the presence of Epi/NE and its antagonist through qRT-PCR. Two constitutive genes, *flaB* and the 16S rRNA gene (*rrs2*), of *Leptospira* were included in the

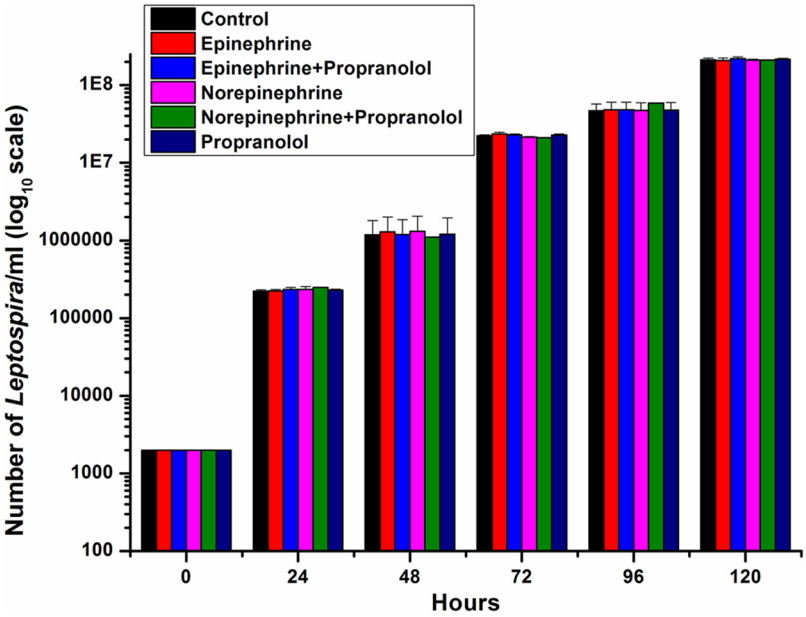

FIG 1 Effects of catecholamines and inhibitor on growth of *Leptospira*. *L. interrogans* Lai cultures were incubated and grown in the presence of Epi, NE, the β-antagonist propranolol (PO), or the catecholamines and PO combined. Spirochete cultures were monitored daily, and spirochete numbers were determined by counting under 40× phase-contrast microscopy. Each treatment showed growth similar to those of untreated control cultures, and at no time point was a significant difference detected among any group. Results are indicative of those from two independent experiments.

study to select for the best gene that can be implemented for normalizing gene transcription data obtained through qRT-PCR. There was no significant change in the transcription of the 16S rRNA gene (threshold cycle [C_T] values) in comparison to that of *flaB* in any of the experimental samples analyzed by qRT-PCR. Therefore, the transcripts of target genes of *Leptospira* grown under IVCL supplemented with catecholamines were normalized with 16S rRNA transcripts using the $2^{-\Delta\Delta CT}$ method; the transcripts of each normalized gene are represented as the number of gene transcripts per 1,000 copies of the 16S rRNA gene. It is to be noted that there are two 16S rRNA genes in *Leptospira*, and in this study, *rrs2* was chosen because it gave higher C_T values than *rrs1*.

In statistical analyses, there were 7 genes (*LB047*, *LB186*, *LB191*, *LA0616*, *LA3263*, *LA3307*, and *LA3961*) out of 41 that showed significant ($P < 0.05$) differential transcription in the presence of Epi/NE which was restored to the basal level (control) in the presence of its antagonist (Fig. 2A). Encouraged by this finding, it was interesting to evaluate if the effect of catecholamine was universal to other pathogenic serovars of *Leptospira*. A nucleotide BLAST to determine the sequence identity of the genes in pathogenic serovars Lai and Copenhageni from the KEGG GENES database was performed (19). The genes which were significantly upregulated or downregulated in serovar Lai were found to be present in serovar Copenhageni, with 99% gene sequence identity, as shown in Table 1. Therefore, under similar experimental conditions, the effect of catecholamines on *L. interrogans* serovar Copenhageni gene transcripts was analyzed. As expected, gene transcripts of these selective 7 genes in serovar Copenhageni also showed a similar trend of differential transcription in the presence of catecholamines or its antagonist (Fig. 2B). The fold changes of transcripts of these 7 genes in the presence of Epi/NE were calculated with respect to basal-level expression (control) for both serovars of *Leptospira* (Fig. 2C and D). It was observed that the two genes (Lai/Copenhageni: *LB191/LIC20151* and *LA3307/LIC10841*) were differentially transcribed in the presence of both Epi and NE, whereas 5 genes responded to the presence of either Epi (*LB047/LIC20035*) or NE (*LA0616/LIC12966*, *LA3961/LIC13166*, *LA3263/*

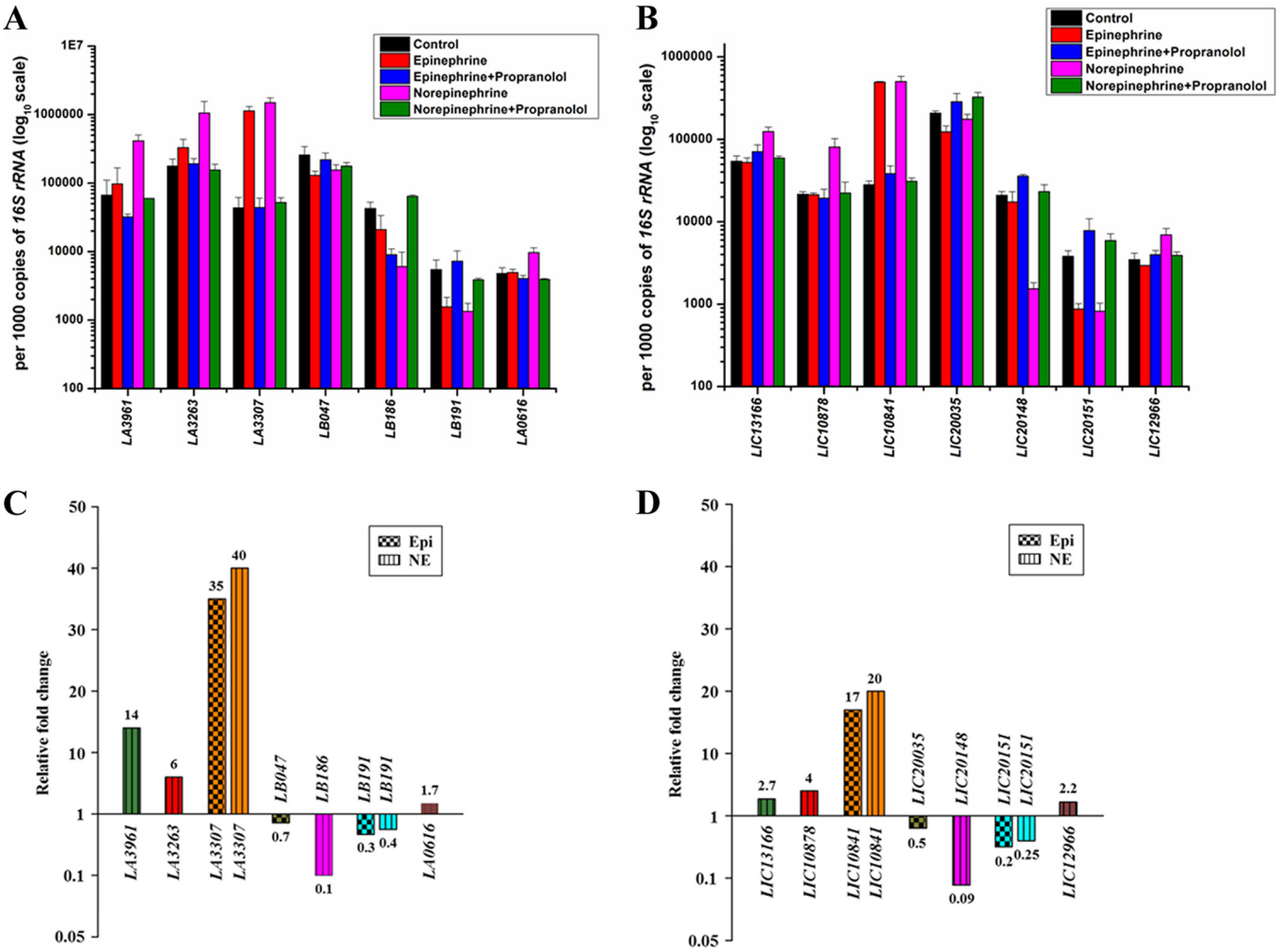

FIG 2 Effects of catecholamines and inhibitor on the selective gene transcripts of *Leptospira*. Transcript analyses of the 7 genes encoding OMPs out of 41 by qRT-PCR of the cDNA synthesized from *Leptospira* grown in the presence of catecholamines and inhibitors (500 μM) were conducted. The gene transcription was calculated based on threshold cycle (C_T) values by use of the $2^{-\Delta\Delta CT}$ method and normalized against 16S rRNA values. (A) The genes of *L. interrogans* serovar Lai that showed significant differential transcription. The differential transcription of seven genes in the presence of catecholamines was restored to the basal level on addition of its inhibitor propranolol describing the specific role of the catecholamines in modulating gene transcription. (B) The differential transcription of 7 orthologous genes of *L. interrogans* serovar Copenhageni. The patterns of differential transcription of 7 orthologs of Copenhageni to serovar Lai are very close. (C and D) The fold change in gene transcription of *L. interrogans* Lai and *L. interrogans* Copenhageni, responding significantly to catecholamines. Each gene is represented by a unique colored bar along with fold change value at the apex of each bar. Error bars indicate the SDs from 2 independent qRT-PCR analyses.

LIC10878, and *LB186/LIC20148*). Additionally, three genes (*LB047/LIC20035*, *LB186/LIC20148*, and *LB191/LIC20151*) in the presence of Epi/NE showed downregulation in its transcription and four genes (*LA0616/LIC12966*, *LA3961/LIC13166*, *LA3263/LIC10878*, and *LA3307/LIC10841*) were upregulated. The gene transcript (normalized) data that showed statistically insignificant differential transcription (32 genes) of the spirochetes were segregated in three independent clusters based on copies of gene transcripts (see Fig. S1A, B, and C in the supplemental material). Among the 41 selected genes, 2 genes (*LB194* and *LA3340*) were excluded from the study because they showed very high fluctuations in their C_T values.

The impact of gene transcription represented here was achieved by growing spirochetes in the presence of catecholamine at a concentration of 500 μM. In contrast, numerous studies on other bacteria have been performed by supplementing catecholamine at a physiological concentration (50 μM) reflecting host gastrointestinal tract stress conditions (20, 21). Therefore, another independent experiment was performed to analyze the effect of catecholamine at 50 μM on the 7 differentially transcribed

TABLE 1 Comparative analyses of the gene sequence identities of the differentially regulated genes of *Leptospira* serovars Lai and Copenhageni in the presence of catecholamines

Serovar	Locus	Gene	No. of bases in CDS[a]	No. of identical bases/total (% identity)
Lai	*LA0616*	*lipL41*	1,068	1,065/1,068 (99)
Copenhageni	*LIC12966*		1,068	
Lai	*LA3961*	*ompL36*	912	910/912 (99)
Copenhageni	*LIC13166*		921	
Lai	*LB186*	*hol*	678	676/678 (99)
Copenhageni	*LIC20148*		678	
Lai	*LB191*	*hbpA*	2,133	2,131/2,133 (99)
Copenhageni	*LIC20151*		2,133	
Lai	*LA3307*	*rfe*	1,047	912/915 (99)
Copenhageni	*LIC10841*		915	
Lai	*LA3263*	Hypothetical	1,224	1,221/1,224 (99)
Copenhageni	*LIC10878*		1,224	
Lai	*LB047*	Hypothetical	1,320	1,313/1,320 (99)
Copenhageni	*LIC20035*		1,323	

[a]CDS, coding sequence.

genes of spirochetes (Fig. S2). The transcript results indicated a similar response in *L. interrogans* serovar Lai to the presence of catecholamine at 50 μM (Fig. S2).

Molecular characterization of hypothetical protein LIC20035/LB047. Among the seven genes that were significantly differentially expressed in the presence of catecholamine(s), *LIC20035/LB047*, encoding hypothetical outer membrane protein in *Leptospira*, was selected for further characterization. Protein BLAST was performed to identify orthologs of LIC20035 in the pathogenic, intermediate, and saprophytic strains of *Leptospira*. As is evident from Table 2, LIC20035 is better conserved (88 to 99% amino acid sequence identity) across pathogenic leptospires than across intermediate (62 to 64%) and saprophytic (39 to 40%) strains. Oligomers designed for *LIC20035* were used to analyze the existence of this gene by PCR in various available pathogenic (*L. interrogans* serovars like Copenhageni, Lai, and Canicola) and saprophytic (*L. biflexa* serovar Patoc) forms of *Leptospira*. Using PCR, amplification of 1,275 bp confirmed the existence of *LIC20035* in various pathogenic spirochete serovars, *viz.*, Copenhageni, Lai, and Canicola of *Leptospira*. Interestingly, no amplicon was detected in nonpathogenic serovar Patoc (Fig. 3A). However, amplification of *flaB* gene (852 bp) could be seen in all pathogenic and nonpathogenic serovars of *Leptospira* (Fig. 3A).

TABLE 2 Comparative analyses of the protein sequence identity of LIC20035 in the different strains of *Leptospira*

Leptospira species	Type of strain[a]	Query coverage (%)	Identity (%)	NCBI accession no.
L. kirschneri	++	99	99	WP_004765841.1
L. noguchii	++	99	96	WP_061247126.1
L. santarosai	++	99	90	WP_004475508.1
L. weilii	++	99	90	WP_002999236.1
L. alexanderi	++	99	90	WP_078124341.1
L. alstonii	++	99	90	WP_020775287.1
L. kmetyi	++	99	90	WP_010572225.1
L. mayottensis	++	99	89	WP_002764046.1
L. borgpetersenii	++	99	88	WP_011671265.1
L. wolffii	+	99	64	WP_016545620.1
L. fainei	+	99	64	WP_016551189.1
L. inadai	+	99	63	WP_010410057.1
L. broomii	+	99	63	WP_010568341.1
L. licerasiae	+	98	62	WP_008589084.1
L. wolbachii	−	99	40	WP_015682912.1
L. biflexa	−	100	39	WP_012476493.1

[a]++, pathogenic; +, intermediate; −, saprophytic.

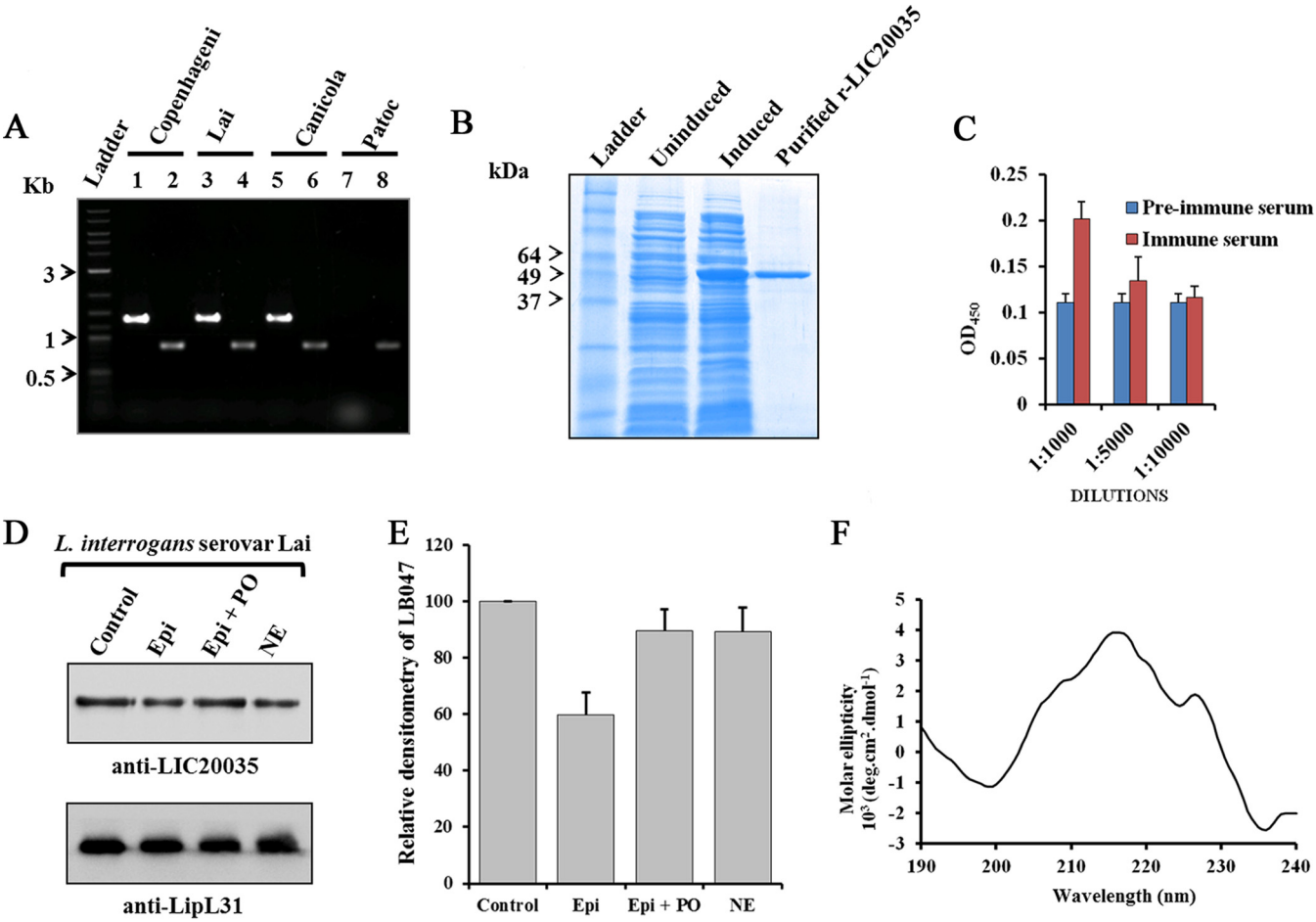

FIG 3 Characterization of hypothetical protein LIC20035/LB047. (A) PCR for *LIC20035* gene using genomic DNA of pathogenic (Copenhageni, Lai, and Canicola) and nonpathogenic (Patoc) serovars of *Leptospira*. An amplicon size of 1,275 bp by agarose gel electrophoresis confirmed the existence of the *LIC20035* gene in pathogenic serovars (lanes 1, 3, and 5). In contrast, no amplification of *LIC20035* was observed in the nonpathogenic serovar of *Leptospira* (lane 7). Primers of the constitutive *flaB* gene were used as a positive control for determining the quality of DNA (lanes 2, 4, 6, and 8). (B) Induction and purification of recombinant LIC20035. The CDS of *LIC20035* of *L. interrogans* Copenhageni L1-130 was cloned without its signal peptide sequence in the pET28a vector and expressed in BL21 *E. coli*. Induction of r-LIC20035 expression was done using 0.5 mM IPTG, and its purification was performed using Ni-NTA column chromatography under hybrid conditions. The uninduced and induced lysates of *E. coli* BL21 along with the purified recombinant LIC20035 are shown on an 12% SDS-PAGE gel stained with Coomassie. (C) Generated polyclonal antibody titer raised against r-LIC20035. The pooled mouse immune serum obtained 10 days after a second booster dose was used to calculate the titer of polyclonal antibodies generated against r-LIC20035 using ELISA. Serum obtained before the immunization of r-LIC20035 antigen was used as a control for evaluation of antibody titer, and data are presented as means ± SEMs from two independent experiments. (D) Immunoblot demonstrating the differential expression of LB047 in *L. interrogans* serovar Lai grown in the presence of catecholamines and inhibitor. There was a repression of LB047 in the presence of Epi alone in comparison to control and NE, which was restored to the basal levels when the organism was grown along with its inhibitor, propranolol. Anti-LipL31 was used as a control to demonstrate equal loading of *Leptospira* lysates. (E) Relative densitometry of the immunoblot shown in panel D was calculated from two independent experiments and normalized with the band intensity of LipL31 using Image Lab software. The repression of LB047 was evident in the presence of Epi, and the effect was restored to the basal level using its inhibitor, PO. (F) Far-UV circular dichroism (CD) of r-LIC20035. The CD spectra are depicted in the range of 190- to 240-nm wavelengths showing a predominant signal of β-strand. CD spectra are shown as averages from 3 scans with a scanning speed of 100 nm/min.

The CDS *LIC20035* was cloned without its predicted signal peptide using genomic DNA of *L. interrogans* serovar Copenhageni and overexpressed in *E. coli* BL21(DE3) cells. The overexpressed r-LIC20035 was purified using Ni affinity column chromatography (Fig. 3B). Subsequently, the purified recombinant protein was used to generate antibodies in BALB/c mice. The polyclonal antibodies generated against r-LIC20035 showed an endpoint titer of 1:5,000 (Fig. 3C). The immunoblot of *L. interrogans* Lai grown in the presence of catecholamines and the antagonist demonstrated that the generated polyclonal anti-LIC20035 was able to recognize LB047 at the expected size of 50 kDa. As expected, there was a repression of the LB047 in the presence of Epi in comparison to the basal expression in the control (Fig. 3D). Interestingly, the expression of LB047 in the spirochetes grown in the presence Epi and its antagonist combined was

considerable, of the same magnitude as that of the control. The consistent expression of antigen LipL31 in the host and under IVCL has been previously described (22). Thus, the same nitrocellulose membrane probed with anti-LipL31 demonstrated equal loading of all the experimental samples (Fig. 3D). In the same context, the relative densitometry of LB047 expression obtained from immunoblot illustrated LB047 to be repressed in the presence of Epi and its expression was restored to the basal level in the presence of the antagonist, PO (Fig. 3E). Further, to understand the function of LIC20035, the secondary structure of the purified r-LIC20035 was evaluated by circular dichroism (CD). The CD spectroscopy data showed maxima at a wavelength of 215 nm and minima at 200 nm (Fig. 3F). The spectroscopic data analysis using the program K2D2 (23) revealed 2% α-helix and 52% β-sheet. This was in agreement with the theoretical secondary structure of r-LIC20035, which was predicted as 0% α-helix and 66% β-sheet using the program PSIPRED.

LIC20035 is a predominant outer membrane surface-exposed protein. Cellular localization of LIC20035 was assessed by Triton X-114 detergent solubilization and phase partitioning. Solubilizing *Leptospira* in 1% Triton X-114 yields a detergent-insoluble fraction known as the protoplasmic cylinder (PC) fraction and a detergent-soluble fraction (24). The Triton X-114-soluble fraction is resolved into two phases by heating above the cloud point (37°C) of this detergent, resulting in separation of the detergent hydrophobic (D) phase from the detergent-poor aqueous (A) phase (25). In fact, previous cellular localization studies of *Leptospira* (24, 25) show that the outer membrane lipoproteins separate into the Triton X-114 detergent phase, while periplasmic proteins separate into the aqueous phase of the soluble fraction. The immunoblot using polyclonal anti-LIC20035 detected LIC20035 to be predominantly present in the detergent phase of the Triton X-114; however, a small amount of the protein could also be traced in the aqueous phase (Fig. 4A). Additionally, to validate the Triton X-114 phase separation experiment, anti-LipL32 was used as a positive control to detect LipL32, which was reported to be present only in the detergent phase (25). A conspicuous band of LipL32 observed only in the detergent phase of the *Leptospira* lysates by immunoblotting underpins experimental procedures for separating the membrane-bound proteins (Fig. 4B).

To validate that LB047 is a surface-exposed membrane protein; a protease accessibility assay was performed for *L. interrogans* serovar Lai. The integrity of the spirochete cell wall during proteinase K treatment was confirmed by performing enzyme-linked immunosorbent assay (ELISA) using antiserum against LipL31, a previously described cytoplasmic protein (25). Similarly, an antibody against a known surface-exposed protein, OmpL54 (26), was used as a positive control. After 1 to 5 h of treatment with proteinase K, LB047 showed a decrease in recognition with its antiserum, similar to the positive control, OmpL54 (Fig. 4C). Around 50% reduction in LB047 recognition was observed after 5 h of protease treatment. In contrast, the absence of significant reduction in reactivity with anti-LipL31 shows that the integrity of the spirochete membrane was not compromised during the proteinase K assay (Fig. 4C). Taken together, the results of cellular localization using Triton X-114 and proteinase K assay suggest LB047/LIC20035 to be a surface-exposed membrane protein.

LIC20035 is detected by antibodies of human and bovine leptospirosis serum. As LIC20035 is localized in the outer membranes of spirochetes, it was interesting to evaluate its serological recognition using immunoglobulins of *Leptospira*-infected hosts. The recombinant proteins (Loa22 and LIC20035) were used to coat microtiter plates and were probed with human or bovine serum which tested positive for leptospirosis ($n = 50$) by the microscopic agglutination test (MAT). As a control, serum samples of human ($n = 15$) and bovine ($n = 10$) that tested negative for leptospirosis by the MAT were included in the serological assay. The average optical density (OD) for serological detection of LIC20035 antigen in humans (Fig. 5A) and bovine serum (Fig. 5C) were heterogeneous (0.5618 and 0.4449, respectively). Moreover, the difference between the absorbances obtained for Loa22 (0.6404) and LIC20035 (0.4449) for bovine infected serum samples was very high (Fig. 5C and D). The calculated sensitivity and

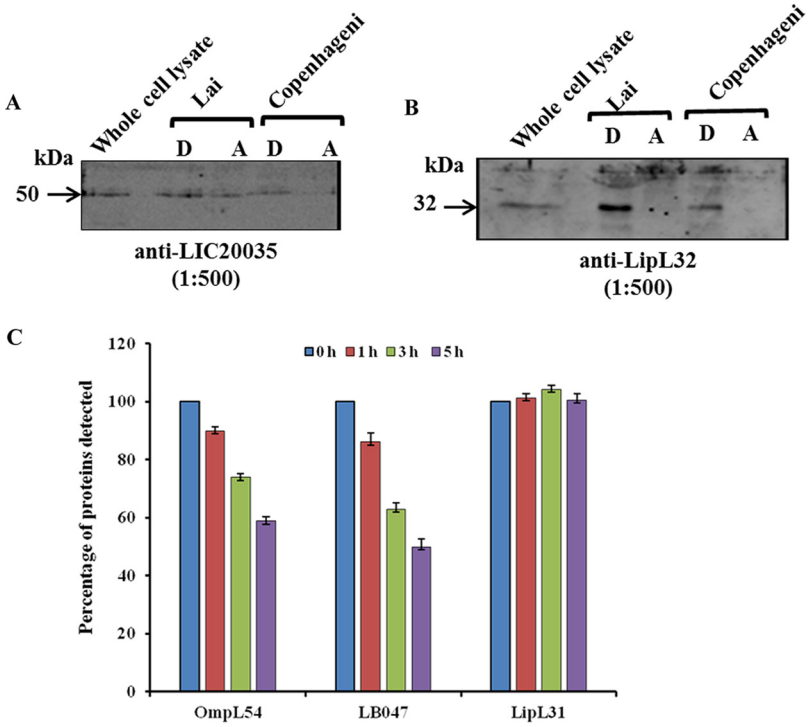

FIG 4 Cellular localization of LIC20035/LB047. (A) Triton-X-114 phase partitioning of spirochete proteins. Spirochete lysates were subjected to Triton X-114 phase partition of aqueous (A) and detergent (D) phases. The aqueous- and detergent-phase fractions were resolved by 12% SDS-PAGE and immunoblotted with anti-LIC20035 serum. The major portion of LIC20035 was in detergent phase and partly in aqueous phase in both serovars Lai and Copenhageni. Whole-cell lysate of serovar Copenhageni in lane 1 was used as a molecular marker for LIC20035. (B) Immunoblot to validate the Triton X-114 phase partition analysis. LipL32 is a known outer membrane lipoprotein and is exclusively present only in the detergent phase of Triton X-114. Anti-LipL32 (1:500) detected LipL32 only in the detergent phase (D) fraction of serovars Lai and Copenhageni. Whole-cell lysate of serovar Copenhageni in lane 1 was used as a molecular marker for LipL32. (C) Proteinase K accessibility assay of *L. interrogans* Lai for surface-exposed LB047. Spirochetes were incubated with 25 μg of proteinase K at various time intervals up to 5 h. The spirochete suspensions were washed with PBS and used to coat a microtiter plate. Using ELISA, a drastic decrease in the signal for LB047 reactivity with its antiserum was observed after 1 to 5 h of proteinase K treatment, similar to the case with OmpL54, a known outer membrane protein. LipL31 was used as a control to check the cellular integrity of spirochetes during treatment with proteinase K. Error bars represent SDs from the three replicates. Statistical analysis was performed by Student's *t* test by comparing the signals obtained for 0 h and other time points of treatment with proteinase K ($P < 0.05$).

specificity of serological detection of LIC20035 by bovine/human serum were 100/98% and 100/100%, respectively. In contrast, Loa22 showed sensitivity and specificity of 100/100% with bovine/human serum (Fig. 5B and D).

LIC20035 binds to extracellular matrix components of host. The extensive study of the attachment of *L. interrogans* to extracellular matrices of hosts gave a clue to the existence of several adhesion molecules (14, 15, 27). Thus, ELISA was performed to study the interaction of r-LIC20035 with ECM components. r-LIC20035 along with r-Loa22 was allowed to bind with the immobilized ECM components on a 96-well plate with bovine serum albumin (BSA) and the highly glycosylated serum protein fetuin as negative controls. r-LIC20035 showed significant binding ($P < 0.001$) with all the ECM components, in contrast to Loa22 (Fig. 6A). The highest affinity of r-LIC20035 was seen with collagen type I and chondroitin sulfate. Additionally, quantitative analyses of the binding of r-LIC20035 with each of these ECM components are shown in Fig. 6B. A dose-dependent saturable binding was observed with increasing concentrations of the recombinant protein (0 to 8 μM) over a fixed amount of the ECM components (1 μg). The r-LIC20035 protein interacts with the host ligands in a dose-dependent and saturable fashion, with calculated K_Ds (equilibrium dissociation constants) of 200 nM,

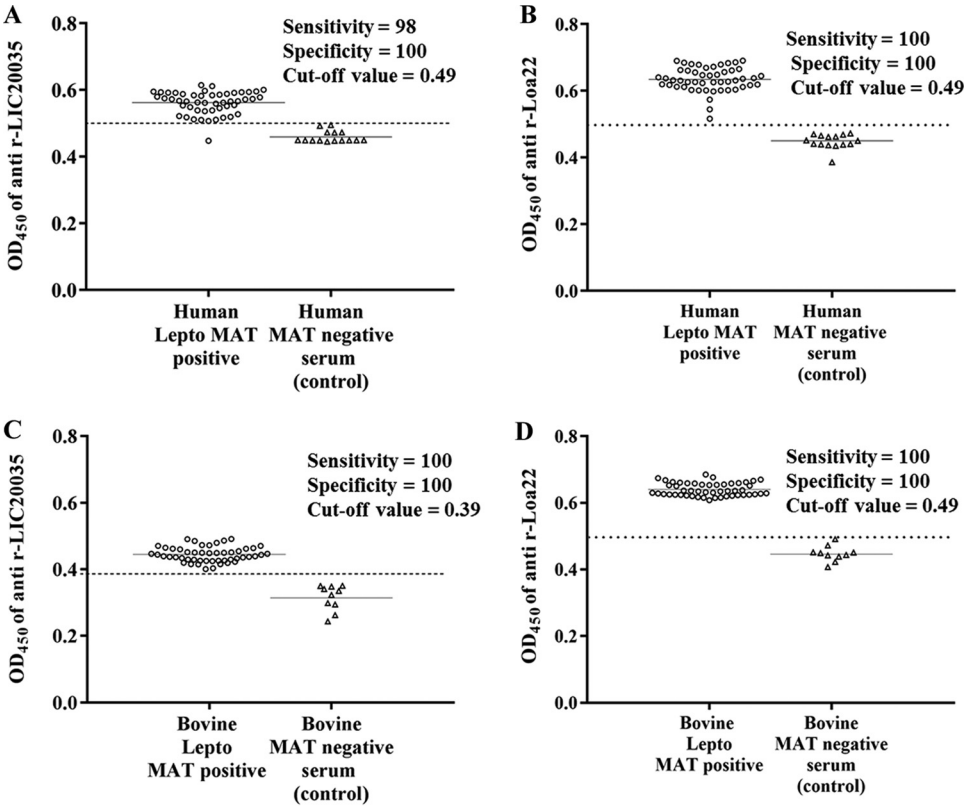

FIG 5 Recombinant LIC20035 is recognized by leptospirosis-positive sera. Enzyme-linked immunosorbent assay (ELISA) was performed to detect r-LIC20035 or r-LIC10191 using the sera of humans (1:100) and bovines (1:100) testing MAT positive ($n = 50$) and control serum testing MAT negative for leptospirosis. The cutoff value of the assay was derived from the mean of the control group plus 2 SDs for each recombinant antigens (dotted black lines). The mean of each group is represented by black horizontal lines. (A) ELISA to detect r-LIC20035 (400 ng/well) using human sera testing positive for leptospirosis. The sensitivity and specificity of the assay were 98% and 100%, respectively. (B) ELISA to detect r-LIC10191 (400 ng/well) using human sera testing positive for leptospirosis. Recombinant LIC10191 (r-Loa22) (400 ng/well, an amount equivalent to r-LIC20035) was used to scale the recognition capacity of human sera. (C) ELISA to detect r-LIC20035 (400 ng/well) with bovine sera testing positive for leptospirosis. The sensitivity and specificity of the serological assay were both 100%. (D) ELISA to detect r-Loa22 with bovine sera testing positive for leptospirosis. Recombinant Loa22 (400 ng/well, an amount equivalent to r-LIC20035) was used to scale the recognition capacity of bovine sera.

250 nM, and 200 nM for collagen type I, chondroitin sulfate A, and chondroitin sulfate B, respectively.

DISCUSSION

A wide range of pathogenic bacteria, like *Salmonella* (28), *Helicobacter* (29), and *Staphylococcus* (30), have been found to be responsive to the stress hormones. However, very little is known about the spirochete response to stress hormones in mammalian hosts. Therefore, in the present study, the effects of stress hormones on the transcription of selective membrane proteins of *Leptospira* were investigated. The typical concentrations of stress hormone used in various studies of microbe-hormone chemical communication were in the range of 50 to 500 μM, which is equivalent to the physiological concentration detected in the host (5, 30). In this study, catecholamines or the antagonist, alone or in combination, did not alter the rate of growth of *Leptospira* under IVCL at 29°C. These results were in agreement with the growth pattern of another spirochete, *Borrelia borgdorferi*, in the presence of the catecholamines and its antagonist propranolol (13). However, for numerous other bacteria, growth rate is also augmented in the presence of catecholamines (31, 32). Apparently, the response of bacteria to catecholamines on its growth rate is variable.

It is evident from the present work that *Leptospira* in the presence of stress

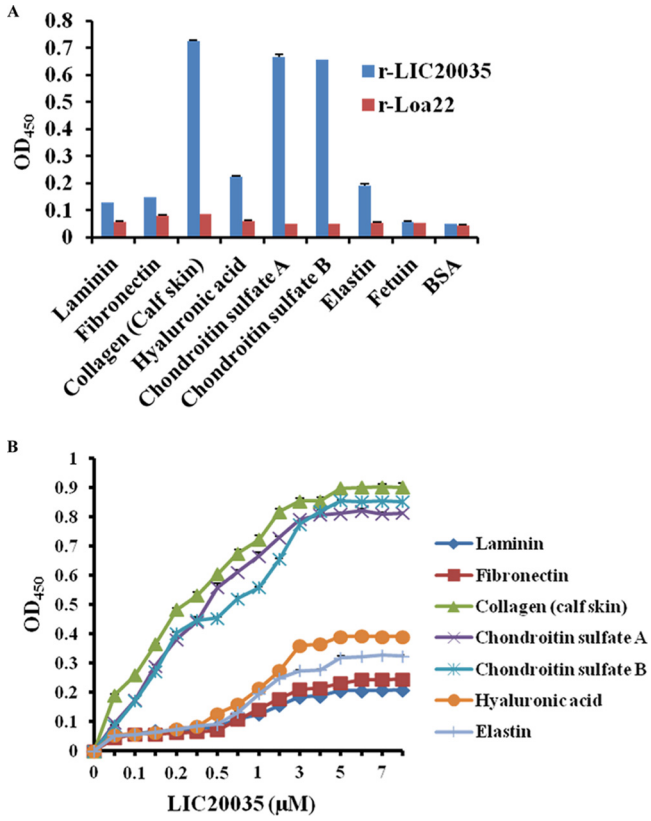

FIG 6 Recombinant LIC20035 binds to host extracellular matrix components. (A) ELISA depicts r-LIC20035 interacts with extracellular matrix (ECM) components, laminin, fibronectin, collagen (calf skin), hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, and elastin. Bovine serum albumin (BSA) and the highly glycosylated serum protein fetuin were used as controls for nonspecific binding. Loa22 was included as a negative control for nonspecific binding with the ECM components. Recombinant LIC20035 exhibited significant binding with all ECM components compared to fetuin or BSA ($P < 0.001$) and with a higher affinity toward chondroitin sulfate and collagen. In contrast, Loa22 showed moderate binding with all the ECM components used in this study. Results are indicative of those from two independent experiments. (B) Dose-dependent binding of the LIC20035 to ECM components. A microtiter plate coated with 1 μg of a specific ECM was incubated with increasing concentrations (0 to 8 μM) of r-LIC20035. Binding of LIC20035 was measured using anti-LIC20035 serum at an appropriate dilution. The mean absorbance values of r-LIC20035 binding to ECM in two experiments at 450 nm are shown.

hormones can modulate its gene transcripts selectively and the effect can be inhibited in the presence of their antagonist. The understanding of the mechanism of such gene modulation in spirochetes due to catecholamine is still in its infancy; however, Sperandio and his coworkers have reported that the catecholamines can induce expression of virulence factors of *E. coli* O157:H7 by mimicking the action of the autoinducer 3 (AI-3) quorum sensing (QS) system in bacteria (33). AI-3 and Epi/NE are thought to be recognized by the same two-component histidine kinase (HK) receptor (QseBC) of *E. coli* O157:H7 (34). We speculate that a similar form of interaction with mammalian catecholamine and HK receptor might play a role in differential transcription of *Leptospira* genes. Using the PSORT program, it is estimated that *Leptospira* possesses 29 HK two-component receptors, out of which 19 are predicted to be located in the inner membrane, 9 in the cytoplasm, and 1 in the periplasm of *Leptospira* (35). Nevertheless, understanding the *Leptospira* mechanism of differential transcription of genes in response to catecholamine is warranted for future studies.

Catecholamines have been reported to bind to the ferric iron binding proteins of host (36) and facilitate the release of iron for microbial uptake. Therefore, 10 genes (*LA1005*, *LA1796*, *LA4253*, *LA0634*, *LB194*, *LB187*, *LB191*, *LB183*, *LA2579*, and *LB186*) of *Leptospira* involved in iron uptake (37) were included in this study. Our results indicate that only two genes of *Leptospira* involved in iron uptake (*LB186* and *LB191*) were

repressed in the presence of catecholamines. The possible reason for such a discrepancy in gene transcription may be a difference in the availability of iron source under *in vitro* and *in vivo* conditions (38). In other words, the available iron in the culture medium, EMJH medium, exists as free Fe^{2+} (39, 40), whereas in the host it is in a bound form.

Besides iron uptake regulators, two genes, namely, *LA3961/LIC13166* (*ompL36*) and *LA0616/LIC12966* (*lipL41*), were upregulated in the presence of NE. Leptospire outer membrane lipoprotein OmpL36 is characterized as a flagellar component of both pathogenic and nonpathogenic strains of *Leptospira* (26). *Leptospira* LipL41, the third most predominant outer membrane lipoprotein (41), is absent in the saprophytic strains (42). The trend of upregulation of *ompL36* transcripts in the presence of NE was possibly a token toward the enhanced motility of the bacteria in the host during stress conditions, leading to dissemination of spirochetes to various host tissues. Our finding that OMPs (LipL41 and OmpL36) are differentially regulated due to host hormonal stress is in agreement with an erstwhile microarray study in which *lipL41* and *ompL36* were highly repressed in the presence of macrophages (38).

The molecular function of the differentially expressed *rfe* gene (*LA3307/LIC10841*) has been predicted to be involved in lipopolysaccharide (LPS) synthesis (UniProtKB accession number Q72U23). This *rfe* gene under our experimental conditions was found to be severalfold (35-/40-fold) upregulated in the presence of Epi/NE. Therefore, understanding the physiological requirements and mechanism for *rfe* sensing of such biochemicals will be an appealing subject of future study. In addition, two hypothetical proteins, namely, LB047/LIC20035 and LA3263/LIC10878, were found to be differentially regulated in the presence of Epi and NE, respectively. This gene regulation was endorsed by the recent report of differentially regulated genes of *Leptospira* grown inside a dialysis membrane chamber or in the presence of macrophages (22, 38).

Among the differentially regulated genes, *LIC20035/LB047* was further characterized because it codes for a conserved hypothetical protein in *Leptospira*. Additionally, LIC20035 orthologs are more conserved among pathogenic leptospires than the intermediate or saprophytic strains. Interestingly, many known leptospiral virulent OMPs, like Loa22, have orthologs in saprophytic strains of *Leptospira*, and therefore, the absence or presence of orthologs in the saprophytes may not be an accurate criterion for predicting a gene to be virulent (43).

The polyclonal antibodies generated against r-LIC20035 were able to recognize native LIC20035 expression predominantly in the detergent phase of Triton X-114. Similarly, a cellular localization experiment using the protease accessibility assay showed LB047 to be surface-exposed membrane protein. However, for some surface-exposed proteins, the proteinase K cleavage sites are inaccessible due to steric hindrance by LPS at the surface of *Leptospira* (26). A previous study on LIC20035 (demarcated as NT03LIA0039, TIGR locus) from elsewhere (44) described LIC20035 to be present in the Triton X-114 fraction of IVCL and was detectable in the immunoblot using antibodies raised against *Leptospira* outer membrane vesicles (OMV). Nevertheless, in the same study LIC20035 was not recognized in the Triton X-114 fraction of spirochetes isolated from infected guinea pig liver (44). The spirochete expression of LIC20035 in guinea pig liver may be due to the multiple effects of host factors; however, this is in agreement with the LIC20035 repression in the presence of Epi.

The seroreactivity of LIC20035 against MAT-positive serum samples of humans/bovines demonstrates that a humoral immune response was generated against LIC20035 during natural infection of *Leptospira* in the host. Recognition of LIC20035 by the hosts during infection suggests that LIC20035 could be a useful serodiagnostic antigen. As OMPs are the primary bacterial components that interact with host cells, targeting newly identified OMPs for development of recombinant vaccines could be rewarding. To date, several OMPs of *Leptospira* have been reported, but none of them have been made commercially available as a highly effective vaccine candidate (45). Considering the wide variety of serovars of *Leptospira* and their host specificity, it is tempting to screen more protective antigens in order to develop a multicomponent

vaccine. Recently, LIC20035 expression was described to be undetectable using serum of rats which were chronically infected with *Leptospira* (44). This may also indicate that the expression of *Leptospira* LIC20035 varies depending upon host chemical signals. The ECM components of the host facilitate adhesion with different microbial proteins, and such interactions are essential for microbial pathogenicity (46). The binding assay of r-LIC20035 with host ECM components showed preferential higher affinity for collagen and chondroitin sulfate. The measured affinity of r-LIC20035 for collagen was near that of LIC13143 (TlyC) (47) but higher than that of LIC11352 (LipL32) (48). Additionally, r-LIC20035 binding to chondroitin sulfate is corroborated by the previous report of *L. interrogans* adhering to chondroitin sulfate (14). The chondroitin sulfate component is one of the most abundant cell surface glycosaminoglycans (GAGs) that mediate bacterial attachment to the host cell surface (49). As LIC20035 is found to be a surface-exposed membrane protein expressed during infection and its recombinant form adheres to more than one host ECM component, it is liable to participate in mediating attachment to host tissues as previously reported for other proteins of spirochetes, like Len (50) and Lsa63 (51). This study mimicked the possible means by which one of the host factors catecholamines is adapted by *Leptospira* to disseminate in various host tissues. Future work on host-pathogen chemical signaling is warranted for development of a wide range of disease intervention strategies.

MATERIALS AND METHODS

Bacterial strains, media, and growth condition. The bacterial strains *L. interrogans* serovar Lai and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were obtained from Indian Council of Medical Research (ICMR), Regional Medical Research Centre, Port Blair, Andaman and Nicobar Island, India. Spirochetes were grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium (Difco) maintained at 29°C with 10% heat-inactivated rabbit serum (56°C for 30 min) unless otherwise stated. For growth rate and gene transcription profiling, 2×10^3 spirochetes were seeded per ml of EMJH medium. To this, catecholamines (Epi or NE; Sigma, USA) and their antagonist propranolol (PO) or an equivalent volume of a vehicle (control) were added in combination or alone to a final concentration of 500 μM until growth reached log phase, as described before (52). The spirochetes were identified and counted every 24 h for 5 days under phase-contrast microscopy (CX41; Olympus) using a Petroff-Hausser cell counting chamber as described elsewhere (13).

Nucleic acid isolation and real-time RT-PCR. Total RNA of spirochetes were isolated from the log-phase of the growth curve in the presence and absence of catecholamines or the antagonist, using the TRIzol (Invitrogen) method as described elsewhere (53). After DNase (New England BioLabs [NEB]) treatment of total RNA, the quality and quantity of RNA were determined using gel electrophoresis and spectrophotometry. A total of 1 μg of spirochete RNA was converted to its cDNA using Verso cDNA synthesis kit (Thermo Scientific). To rule out genomic DNA contamination, cDNA synthesis was performed without adding the reverse transcriptase as a negative control. The oligonucleotides used for qRT-PCR were designed using the OligoPerfect primer design program (Invitrogen) from the available *L. interrogans* serovar Lai genomic sequence. All qRT-PCR oligonucleotide pairs were designed with the same annealing temperature (60°C) and similar amplicon sizes (100 to 300 bp). Each primer pair was tested for efficiency and nonspecific amplification using SYBR green PCR master mix (Applied Biosystems) and *Leptospira* genomic DNA as a template in a 7500 real-time PCR system (Applied Biosystems). The PCR system was programmed for 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melt curve analyses of the PCR products. The details of the genes obtained from UniProtKB database (54) and the primers used in this study are listed in Table 3. Transcripts of target genes were quantified using $2^{-\Delta\Delta CT}$ method (55) and normalized with the 16S rRNA gene of *Leptospira* (56). The expression profile of the genes regulated by the catecholamines or their antagonist in comparison to the control was determined in two independent experiments to achieve statistically significant results.

Protein overexpression and purification. Predicted CDSs of *LIC20035* (1,275 bp) and *LIC10191* (519 bp) were amplified without the signal peptide sequence by PCR from the available genomic sequence of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. An InstaTA cloning kit (Thermo Fisher Scientific) was used to facilitate gene cloning. Thereafter, *LIC20035* was cloned in the pET28a vector and overexpressed in *E. coli* BL21(DE3) competent cells. The restriction endonuclease site (italicized and underlined) of the oligomers used in cloning of *LIC20035* and *LIC10191* genes in the pET28a vector (Novagen San Diego, CA) is shown in Table 3. The calculated molecular masses of r-LIC20035 and r-LIC10191 (Loa22) are 50 and 22 kDa, respectively.

E. coli BL21(DE3) competent cells were induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in 1 liter of LB medium at 37°C for 4 h. The cell pellet obtained was lysed with cold denaturing lysis buffer (8 M urea, 20 mM Na_3PO_4 [pH 7.8], 500 mM NaCl) and thereafter sonicated for 15 min with 6-s on-and-off cycles. The resulting homogenate was centrifuged at 12,000 $\times$ *g* for 30 min to remove cellular debris. Induced recombinant proteins were purified by affinity column chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resins (Invitrogen) using a protocol published elsewhere (57). Briefly,

TABLE 3 Details of the *Leptospira* genes selected and their oligomers in this study[a]

Gene ID of serovars (Lai/Copenhageni)	Gene name and/or function	Gene size (bp)	Sequence (forward and reverse), 5′–3′
LAr04/LIC11508	*rrs2*, 16S rRNA	1,512	TTATTGCTCGGAGATGAGCC, TTCAGGGTTCCCCCCATT
LA2019/LIC11889	Flagellin (*flaB*)	852	ATGATTATCAATCACAACCTGAGTG, TCAGTATTCCTTTCCGCTTGA
LA1005/LIC12655	Uncharacterized	459	GATTCACAGAGTTATCCTAACTTTCTG, CCTGGTATCGAAATGTCAGTTG
LA1796	Uncharacterized	546	TAGGTGAAGGATTTTCATATCCAAA, AAATCCATCAGCATTACTTCTTAGAT
LA0634/LIC12953	*dppC*	762	TTCTTCTGTGTTTGTTTCCGTTC, AAGCAAGTACAAATCCACTTCCA
LB187/LIC20149	Multidrug efflux transporter	1,209	TACTCTTTTTACTCTTTTATTTCTCCG, CTACGGCCCCTCCTAAAGAA
LB191/LIC20151	TonB-dependent receptor	2,133	CGAAGTCATTTCCCGTAAAAAG, GTCCGGAAATTCTTTCGC
LB183/LIC20147	*fur*, transcription regulator	393	TACGAACTTTCCCGAAAGAATTT, ACACGGTCGCATTGTTTACA
LA2579/LIC11402	*feoB*, ferrous transporter	2,106	TTAGTTTGGGAGGGAGCTCA, TTTCATCGTGACCGCGAC
LB186/LIC20148	Heme oxygenase	678	TCAAGACTATGTGCAAAGAATTCG, TAGGAAATTCGTAAAAAGAAATTCCTT
LA4253/LIC13403	*fepB*	774	TCGGATTTTCAGATATACAATCGG, CTTTCTTTTCCAACCTTCAATCA
LA3064/LIC11030	Putative lipoprotein	1,035	AAACCGATACGGAGTTGTCATC, ATTTTGTGCATAAAGTCTCGTGTTT
LA0426/LIC10373	Putative lipoprotein	1,530	ATAGTTGGAACCTCTAATGGACCA, CCGCGACATTCTTTGGGTA
LA0616/LIC12966	*lipL41*, OM lipoprotein	1,068	GTGAAGGTTCCAGCTTTATCGA, CGCTCCAATCAGATTTCCG
LA2637/LIC11352	*lipL32*, OM lipoprotein	819	GTGAAGGTTCCAGCTTTATCGA, CGCTCCAATCAGATTTCCG
LA3961/LIC13166	*ompL36*	921	AAGAGCAGAAGCGTCGTA, TTGGAGAGTTGGTGGAGTT
LA3778/LIC10464	*ligB*	1,890	ACGGTACCAGTACAACCCTAGAAG, TAGGCCGTTGTATTCTGCTTTT
LA1569/LIC12209	Putative lipoprotein	1,416	GGAGACGAGGATTGGGTCC, GTGTTCCGATACTAGAGGGCTTATT
LA3446/LIC10730	Putative lipoprotein	480	AAAAACGAATCGGACTTTCTCC, ATCCATACATTGTCTTTGTTTTGC
LA3247/LIC10889	*tonB*	591	TCATCCAAGAACCAAACGTTG, GGCTTCATCGGGATAGTCTG
LA3258/LIC10881	TonB-dependent OM receptor	1,674	ATTCTAAAGTGTTTTCTTTGTCGGA, GGTTACCTTTTTTGTTTTTTGTACG
LA3478/LIC10704	Putative lipoprotein	669	CAGACAAGGAACGAGAATCTATTG, AGATTGACCTTTCAATTCCGC
LA3444/LIC10731	Putative lipoprotein	1,260	ACCGGAAGATGCCGATTTA, TTTTGTGACCGAATAAACGCT
LA3501/LIC10686	Putative lipoprotein	1,272	GTATGTCTCCAGCAGATGCTCC, GGGCGTTCGATTAGAAAAATT
LA3440/LIC10734	Putative lipoprotein	795	TTCCGGATTTTGTACAAAATCTTC, ATGATATTCTGCCCATATACGCA
LA3371/LIC10792	*mdoC*, glucan biosynthesis	1,173	CCCTTTTTTTCTTTCTTTCGG, AGAAATTTTCGTTCCAGCTTGT
LA3294/LIC10854	*uppS*, isoprenyl transferase	726	CATAGAGAAGGTGCCCAGG, TGAATCGTATCTAATCGAGTTTCTATA
LA3276/LIC10868	Uncharacterized	1,395	GACTTTTAGGGGTAGCGGGA, TCTTGTCCATTGTTTGTTTCCA
LA3263/LIC10878	Uncharacterized	1,224	CTTCAGCATAAAAAAACCTCTTTTGA, ACGATAAATCCTCCCGCC
LA3262/LIC10879	Putative lipoprotein	480	GATCCTTCCTTACTCAATGCTTCT, TCTACTTTTTCAGTTTTTACACGTACA
LA3230/LIC10906	Uncharacterized	909	TAACGTTCCTCCCGATGTC, AGTGTCCGGATCTCCCAGTA
LA3307/LIC10841	*rfe*, transferase	915	GATCGTCTACGTTTTGTTTGTTGT, AGTTAAAACCTAAAAAACCGAAAACA
LA3210/LIC10920	Putative lipoprotein	756	AACCACTGCAGAATCTATGGTG, TTCCGCTTGGATAAACTGGA
LA3200b/LIC10927	Putative lipoprotein	1,494	CGATTACTATTTCCGGAGCC, TCCGAAGCAGTTATATCCACTC
LA3138/LIC10973	*ompL1*	963	TAAATTGATTACCCTCGATAGAACTAC, CTGCTTTTGTAATACCGCCAG
LA1691/LIC12099	*lipL53*	1,431	AATCAAACTTGGGTCAGACAACTT, TCACGCCGAAGTCATTTATCT
LA3017/LIC11058	*lemA*, putative lipoprotein	597	TTCACAAGCGCAAGCACAG, TCTGTTTCTGGCTACGGTAATTC
LA3434/LIC10739	*amiA*, alanine amidase	1,092	CGTTATGTGCGCTTTGAAGA, TAAAGAATCGGAACGGAAATTTT
LA3394/LIC10774	Putative lipoprotein	1,107	CAAGAATCCGAAAAATCTTCTTCA, TTGAAGGGTTGGTTTGACGT
LB194/LIC20153	Putative lipoprotein	579	CTACTTCCATTCAAACTCTTGTTTACG, AGTATTATAAATCAAAGGTGCATTCTC
LA3340/LIC10821	Putative lipoprotein	783	GCGATTCCAATGCTGGTAC, ACTGTCCCCATATAGATTGACACC
LB047/LIC20035	Hypothetical protein	1,323	TGTAACTCCACTCCTAATGTGGAG, CGATTTTAAACCATCTAACTGTTCAG; CTA*GCTAGC*TGTAACTCCACTCCTAATGTGGAG,[b] CCG*CTCGAG*TTATTTAC AACCTTGCATTTCTCC[b]
LA0222/LIC10191	*loa22*, OmpA lipoprotein	588	GATAGTTACGCTCTTGAA, GATACGATTTGCTGGAAT; CTA*GCTAGC*GCTG AAAAAAAAGAGGAATCCG,[b] CCG*CTCGAG*TTATTGTTGTGGTGCGGAAG[b]

[a]ID, identifier; OM, outer membrane.
[b]The restriction endonuclease site of the oligomer used in cloning of the genes in the pET28a vector (Novagen, San Diego, CA) is italicized and underlined.

N-terminal 6× His-tagged recombinant proteins bound to Ni-NTA resins were washed with denaturing wash buffer (8 M urea, 20 mM Na_3PO_4 [pH 6.0], 500 mM NaCl) and subsequently with native wash buffer (50 mM NaH_2PO_4 [pH 8.0], 500 mM NaCl, and 20 mM imidazole). The recombinant proteins were then eluted out using elution buffer (50 mM NaH_2PO_4 [pH 8.0], 500 mM NaCl, and 250 mM imidazole). Thereafter, the purified proteins were dialyzed (Pierce; protein dialyzing cassette) and concentrated (Corning; Centricon Spin-x-UF) to 0.4 mg/ml in dialysis buffer (50 mM Tris-Cl buffer [pH 8] and 100 mM NaCl) before being stored at −80°C.

CD spectroscopy. Circular dichroism (CD) spectroscopy measurements were performed for r-LIC20035 at room temperature using a Jasco J-815 spectropolarimeter (Japan Spectroscopic, Tokyo) at a scanning speed of 100 nm/min. Far-UV CD spectra were measured using a 5-mm-path-length cell at 0.5-nm intervals. The spectra were presented as an average of 3 scans recorded from 190 to 260 nm and smoothed using a Savitzky–Golay filter (58). The molar ellipticity (Φ) is expressed in degrees $\cdot$ cm^2 $\cdot$ dmol^{-1}. Spectral data were submitted to the K2D2 web server (23), which calculated the secondary structure content from the ellipticity experimental data. The theoretical secondary structure was calculated using PSIPRED v3.3 (59).

Generation of polyclonal antibodies against purified recombinant LIC20035 and Loa22. Antigen r-LIC20035 was used to immunize 4- to 6-week-old female BALB/c mice subcutaneously. About 10 μg per mouse of recombinant protein emulsified in Freund's complete adjuvant (FCA; catalog no. sc-3727; Santa Cruz Biotechnology) was used for primary immunization (5 mice per group). A negative-control group was injected with an equal volume of phosphate-buffered saline (PBS) along with the adjuvant. Immunized mice were further given two booster injections of recombinant protein emulsified in Freund's incomplete adjuvant (FIA; catalog no. 3726; Santa Cruz Biotechnology) at 14 and 24 days of primary immunization. At 10 days after the second booster, blood was collected from each mouse by retro-orbital bleeding and then the mouse was sacrificed using atlanto-occipital dislocation method as described before (57). Sera obtained were pooled for antibody titer analysis by enzyme-linked immunosorbent assay (ELISA) before experimental use. Immunization experiments with mice were performed at the Department of Veterinary Microbiology, College of Veterinary Science, Assam Agriculture University Guwahati, India, after approval by the Institutional Animal Ethics Committee. Antibodies against r-Loa22 were generated in rabbits by outsourcing the purified protein to Abgenex, Bhubaneswar, India.

ELISA for LIC20035 titer determination. A disposable 96-well polystyrene plate was coated with 50 μl of r-LIC20035 (400 ng/well) and incubated overnight at 4°C. It was blocked with 100 μl of 3% bovine serum albumin (BSA) at 37°C for 2 h. After three washings of wells with 200 μl of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), the plate was incubated with 50 μl of mouse anti-LIC20035 of various dilutions (1:1,000; 1:5,000, and 1:10,000) at 37°C for 2 h. Pooled preimmune serum of mice was used as a negative control. After three washings, wells were probed with 50 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000) for 1 h at 37°C. The plate was washed as described above, and binding was detected by adding tetramethyl benzidine (TMB) peroxidase substrate (Thermo Fisher Scientific) for 10 min at 37°C. Final optical density (OD) was taken at a wavelength of 450 nm using an ELISA plate reader (Infinite 200 Pro; Tecan) after the reaction was terminated with 1 M H_2SO_4. The endpoint titer was determined visually, being the highest serum dilution giving a positive color development.

Immunoblot assay. Whole-cell lysates of *L. interrogans* serovar Lai grown in the presence and absence of catecholamines were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane (HiMedia). Membranes were blocked with 5% nonfat dried milk diluted in Tris-buffered saline (TBS; pH 8) containing 0.05% Tween 20 (TBS-T) and probed with anti-LIC20035 (1:500) mouse polyclonal serum for 2 h at room temperature. After being washed, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (1:5,000; Sigma) in TBS for 1 h. The protein reactivity was revealed by adding chemiluminescence substrate (Thermo Scientific; catalog no. 32209) over the nitrocellulose membrane and imaging under Chemidoc (Bio-Rad XRS+). Band densitometry of the LB047 expression obtained from the immunoblot was quantified using Image Lab software (Bio-Rad) as described before (60), and the density values were normalized with LipL31 expression in the immunoblot. The relative densitometry of LB047 expression was calculated in terms of percentage from two independent immunoblot experiments.

Phase separation of integral membrane protein using Triton X-114. Phase separation of the integral membrane proteins of *Leptospira* to localize LIC20035 was performed using Triton X-114 solution as described elsewhere (61). Briefly, 1×10^9 spirochetes grown in EMJH medium were centrifuged ($5{,}000 \times g$) and washed 3 times with PBS. The pellet obtained was sonicated four times with 20-s bursts after suspension in 800 μl of PBS. The suspension was added with 200 μl of 10% Triton X-114 (Sigma; catalog no. X114) and was rocked overnight at 4°C. The insoluble debris was removed by centrifugation at $13{,}000 \times g$ and 4°C for 15 min and placed in a 37°C water bath for 10 min. The suspension was centrifuged for 10 min at $13{,}000 \times g$ at room temperature to separate the detergent (D) and the aqueous (A) phases. The detergent phase (50 μl) was mixed with 1 ml of original buffer at 0°C, rewarmed at 37°C, and spun in a microcentrifuge as before. Later, the final aqueous and detergent phases obtained after three washings were precipitated with 10 volumes of chilled acetone. The aqueous and detergent phases were then resolved by 10% SDS-PAGE before being transferred to a nitrocellulose membrane (Himedia) for immunoblotting.

Protease accessibility assay for the cellular localization of LB047. A proteinase K assay was performed for cellular localization of LB047 using procedures described elsewhere (62). Briefly, suspensions of 15 ml of 6-day-old live *L. interrogans* Lai (2.5×10^8 spirochetes/ml) were harvested and resuspended in 6 ml of PBS containing 25 μg of proteinase K (SRL). Tubes containing 1 ml of resuspended spirochetes were then incubated for 0, 1, 3, and 5 h before the addition of 10 μl of 100 mM phenylmethylsulfonyl fluoride (PMSF) to terminate the proteinase K activity. The suspensions were subsequently pelleted by centrifugation at $5{,}000 \times g$ for 15 min, washed twice with PBS, and resuspended in 1 ml of PBS for performing ELISA using antibodies against OmpL54, LB047, and LipL31. OmpL54 and LipL31 are outer membrane and cytoplasmic leptospiral proteins that were used in this experiment as positive and negative controls, respectively (26, 63). The antibodies against OmpL54 and LipL31 raised in rabbits were obtained from David Haake. For the cellular localization of LB047 by ELISA, 100 μl of the proteinase K-treated leptospires was used to coat the microtest plates and incubated for 16 h at room temperature. The primary antibodies against OmpL54, LIC20035, and LipL31 were used at dilutions of 1:50, 1:500, and 1:1,000, respectively. Goat anti-rabbit/anti-mouse IgG HRP-conjugated secondary antibodies were used at a dilution of 1:5,000. The binding was detected as described for endpoint titer determination.

ELISA for recognition of recombinant LIC20035 using sera positive for leptospirosis. Samples of human and bovine sera ($n = 50$) which had tested positive for leptospirosis through the microscopic agglutination test (MAT) were used for recognition of r-LIC20035 by ELISA. Another antigen, r-LIC10191

(Loa22), a known surface-exposed lipoprotein for leptospirosis diagnosis (64), was taken in an equivalent amount to confirm the ELISA results and to scale the absorbance obtained for r-LIC20035. Microtiter plates were coated in duplicates with equal amounts (400 ng/well) of recombinant proteins (r-LIC20035 and r-Loa22) overnight at 4°C, and thereafter, unbound surface was blocked with 3% BSA at 37°C for 2 h. After 3 washings of the plate with 200 μl of phosphate-buffered saline containing 0.05% Tween 20, wells were probed with human/bovine leptospirosis serum (1:100) at 37°C for 2 h. Microtiter wells were probed with secondary HRP-conjugated anti-human IgG (Genei; catalog no. 62114028001A)/anti-bovine IgG (Sigma; catalog no. A5295) for 1 h (1:5,000) at 37°C, and the binding was measured as described for endpoint titer determination. The cutoff value for antibody reactivity was calculated as described previously (64). Briefly, the average and the standard deviation (SD) were calculated from the MAT-negative serum groups, and the cutoff value of the average + 2 SDs was used for analysis. All the absorbance values obtained from the ELISA which equaled or exceeded the cutoff value were considered positive for infection and used to calculate the percent sensitivity of the assay. The percent specificity of the assay was calculated using the number of samples of the MAT-negative group below the calculated cutoff.

ELISA for binding of r-LIC20035 to host ECM components. Host ligands included in the study were fibronectin (Sigma; catalog no. F4759), laminin (Sigma; catalog no. L2020), bovine skin collagen type I (Sigma; catalog no. C9791), elastin (Sigma; catalog no. E1625), chondroitin sulfates A and B (Sigma; catalog no. C9819/C3788), and hyaluronic acid (Sigma; catalog no. H7630). As negative controls for ligand, fetuin from fetal calf serum and BSA (New England BioLabs) were used. Recombinant Loa22 was used as a negative-control antigen in this assay, as it was previously shown to interact moderately with the tested extracellular matrix (ECM) components (27).The attachment of r-LIC20035 and r-Loa22 to these host ECM components was analyzed by indirect ELISA. Briefly, ELISA plates were coated in duplicates with 1 μg of the solubilized ECM components, BSA (negative-control ligand) and fetuin (highly glycosylated attachment negative-control ligand), in 50 μl of PBS for 2 h at 37°C. Further procedures were followed as described for endpoint titer determination.

Dose-response curves and K_D values. ELISA plates were coated overnight with 1 μg of different ECM components. Each plate was blocked and increasing concentrations of purified r-LIC20035 (0 to 8 μM) were added (50 μl per well in PBS), followed by incubation for 2 h at 37°C. The assessment of bound protein was performed with polyclonal antiserum raised in mice against LIC20035 followed by HRP-conjugated anti-mouse IgG. The ELISA data, when reactions reached a saturation point, were used to calculate the K_D according to a described method (65), following the equation $K_D = (A_{max}[\text{protein}]/A) - [\text{protein}]$, where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration, and K_D is the equilibrium dissociation constant for a given protein concentration (ELISA data point).

Statistical analysis. All results are expressed as means ± standard errors. Student's paired *t* test was used to determine the significance of differences between means, and *P* values of <0.05 were considered statistically significant. Two independent experiments were performed, each one in duplicate or triplicate.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02360-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We have no conflicts of interest to declare.

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