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A Putative Regulatory Genetic Locus Modulates Virulence in the Pathogen *Leptospira interrogans*

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Limited research has been conducted on the role of transcriptional regulators in relation to virulence in *Leptospira interrogans*, the etiological agent of leptospirosis. Here, we identify an *L. interrogans* locus that encodes a sensor protein, an anti-sigma factor antagonist, and two genes encoding proteins of unknown function. Transposon insertion into the gene encoding the sensor protein led to dampened transcription of the other 3 genes in this locus. This lb139 insertion mutant (the lb139$^-$ mutant) displayed attenuated virulence in the hamster model of infection and reduced motility *in vitro*. Whole-transcriptome analyses using RNA sequencing revealed the downregulation of 115 genes and the upregulation of 28 genes, with an overrepresentation of gene products functioning in motility and signal transduction and numerous gene products with unknown functions, predicted to be localized to the extracellular space. Another significant finding encompassed suppressed expression of the majority of the genes previously demonstrated to be upregulated at physiological osmolarity, including the sphingomyelinase C precursor Sph2 and LigB. We provide insight into a possible requirement for transcriptional regulation as it relates to leptospiral virulence and suggest various biological processes that are affected due to the loss of native expression of this genetic locus.

The genus *Leptospira* consists of both saprophytic and pathogenic species, with the latter being the causative agents of leptospirosis. *Leptospira* bacteria are capable of infecting numerous mammals and have been detected in animals and humans on all continents, with the exception of Antarctica (1). The World Health Organization estimates the worldwide leptospirosis incidence in humans to be 0.1 to 1 per 100,000 population in areas with temperate climates, 10 or more per 100,000 population in areas with tropical climates, and as high as 100 per 100,000 population during an epidemic. Leptospirosis manifests a broad range of symptoms, including fever, general feelings of malaise, and jaundice, and in severe cases can lead to liver and/or kidney failure with a mortality rate of 5 to 15% in humans (1).

Leptospira bacteria are zoonotic pathogens that colonize the kidneys of rodents, which can serve as maintenance hosts in the majority of epidemiological settings (1). Transmission to other animal hosts is thought to occur via the shedding of *Leptospira* in the urine of maintenance animals into environmental water reservoirs which can support *Leptospira* viability (1). Subsequent contact of an organism that has incurred an abraded epidermis with the contaminated water increases the risk of leptospiral infection (2). The shift from a water reservoir to animals or humans results in a shift of the *Leptospira* bacteria between drastically different environments, including environments with different temperatures and nutrient supplies and other complex interactions, such as the host immune system and microbial flora. Thus, *Leptospira* must be able to adapt to such disparate changes in environmental conditions to retain viability. To do so, *Leptospira* bacteria likely first sense these variations in their surrounding environments through a complex coordination of response regulators which, upon sensing these alterations, undergo conformational changes. The altered conformation of receptors initiates relay systems which ultimately alter the bacterial transcriptome and proteome so that the organism is more suited for the conditions in the immediate environment. Evidence for altered leptospiral transcription in response to environmental changes has been provided by numerous studies that have demonstrated an altered transcriptome in response to temperature changes (3, 4); depleted iron conditions (5); and exposure to serum (6), host innate immune cells (7), and physiological osmolarity (8) and in mouse and hamster infection models (9).

Well-characterized pathways that regulate the bacterial response to environmental change and virulence include sigma B regulatory networks, such as RsbT/RsbV in *Listeria monocytogenes* (10), RsbU in *Staphylococcus aureus* (11), and RsBV/RsbW in *Bacillus anthracis* (12). Other examples include the RpoS/RpoN system in the spirochete bacterium *Borrelia burgdorferi* (13) and the RpoE system in the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (14). The general mechanism in all these systems includes activation of a receptor that senses a change in the environment, followed by phosphorelay events leading to the activation of sigma factors that ultimately join RNA polymerase to initiate the transcription of target genes.

By means of random insertion inactivation in *Leptospira interrogans* serovar Manilae strain L495, we identified a leptospiral transposon mutant that displayed attenuation in the hamster infection model. Characterization of the insertion site revealed in-

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sertion in chromosome II in a gene (lb139) encoding a sensor protein containing a phosphatase domain. Further analyses of the genetic locus revealed an anti-sigma factor antagonist two genes downstream of lb139. The possibility that transposon insertion into lb139 would disrupt the function of this gene and lead to deregulation of the downstream genes, thereby altering global transcriptional regulation, prompted us to further characterize the lb139 insertion mutant (the lb139$^-$ mutant) strain. Whole-transcriptome analyses revealed decreased numbers of transcripts of 115 genes and increased numbers of transcripts of 28 genes in the lb139$^-$ strain. When these findings were compared to the genome-wide predicted frequencies for genes based on clusters of orthologous groups (COG), our analyses revealed a bias toward the downregulation of genes involved in motility/chemotaxis and genes involved in signal transduction. We present our results for deregulated genes and discuss the role of the potential regulatory locus lb139-lb136 (encompassing genes lb139, lb138, lb137, and lb136) in the context of leptospiral virulence.

MATERIALS AND METHODS

Bacteria and culturing. *Leptospira interrogans* serovar Manilae strain L495, the lb139$^-$ mutant, and lb139$^-$ mutant complemented with lb139 (lb139$^{-/+}$) were grown in EMJH (15, 16) at 30°C with agitation.

Insertion mutagenesis and complementation. Insertion inactivation in *L. interrogans* serovar Manilae strain L495 has been previously described (17). The insertion site was identified by semirandom PCR followed by DNA sequencing (18), and the insertion within lb139 was confirmed via PCR using primers flanking the insertion site. For complementation, lb139 and its native promoter were PCR amplified using primers lb139F (5′-CCAGAGTGACTTTTAATTCATAG-3′) and lb139R (5′-GGTCGACTTATTATAATTTTGGAAATATGCA-3′), which annealed 105 to 83 bp downstream and 320 to 296 bp upstream of the coding region, respectively. The genomic coordinates for lb139 in serovar Manilae, as annotated in MicroScope (http://www.genoscope.cns.fr/agc/microscope/home/index.php), are 4210046 to 4211533, and the annealing coordinates for primers lb139F and lb139R are 4209941 to 4209963 and 4211829 to 4211853, respectively. The amplicon was then ligated into the PCR2.1 TOPO vector (TOPO TA Cloning kit with PCR2.1 TOPO; Invitrogen) according to the manufacturer's instructions and used to transform One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). The plasmid was purified and double restriction digested with KpnI and XhoI, and the gel-purified insert was subsequently ligated into plasmid pAL614 (a gift from Gerald Murray, Monash University, Victoria, Australia), which carries a modified Himar1 transposon containing a spectinomycin resistance cassette. The resulting plasmid was then used to chemically transform *E. coli* SM10 cells, which were subsequently used to transform the serovar Manilae lb139$^-$ strain via conjugation (19). Complementation of the lb139$^-$ strain was confirmed by growth in medium containing spectinomycin and by using primers that PCR amplified a region of the spectinomycin resistance cassette and primers lb139F and lb139R, using genomic DNA as the template. The position of complementation was at nucleotide 181482 in the open reading frame at coordinates 180854 to 183208 encoding a hypothetical protein (la0172). We do not know whether disruption of this gene results in attenuated virulence. However, we have tested independent complemented lb139$^-$ mutants with insertions at different sites and found that their virulence was also attenuated.

DNA extraction, Southern blotting, and genome sequencing. The *Leptospira* lb139$^-$ mutant was cultured to ~5×10^8 ml^{-1}, and DNA was extracted from a total of ~3×10^{10} bacteria. Southern blot analysis was performed as previously described (20) using EcoRI-digested lb139$^-$ mutant DNA and DNA from various other insertion mutants for comparison. Whole-genome sequencing was performed on the lb139$^-$ mutant with the Illumina (San Diego, CA) paired-end sequencing technology by the Plate-Forme Génomique at the Institut Pasteur. Sequencing and library preparation were performed as described by the supplier (NEBNext Ultra DNA library preparation kit for Illumina; New England BioLabs). Briefly, genomic DNA was sheered by nebulization, and fragments were end repaired and phosphorylated. Blunt-end fragments were A tailed, and sequencing adapters were ligated to the fragments. Size selection was performed using AMPure XPbeads (approximately 500 bp), and sized fragments were enriched with 10 cycles of PCR using Q5 High-Fidelity DNA polymerase. Sequencing was performed for 2 × 250 cycles on a MiSeq sequencing platform (Illumina, San Diego, CA) using a paired end (MiSeq reagent kit, version 2 [500 cycles]). Image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline (version 1.7) software. High-quality filtered reads (3,478,082 reads, ~173-fold coverage) were assembled using the CLC Assembly Cell program (CLC bio).

Single nucleotide polymorphisms (SNPs) and insertions and deletions were considered real if these changes were present in all the sequencing reads for a given gene. Changes that were identified in this manner and that resulted in nonsynonymous mutations, insertions, or deletions were further confirmed via PCR amplification using primers flanking the regions with the observed change and subsequent sequencing, using DNA from both the lb139$^-$ mutant and wild-type Manilae L495 for comparison. This approach identified two changes; the first was a nonsynonymous SNP in LMANv1_50006 (GGT to GAT [G to D] in codon 198), which is orthologous to la0025 encoding FliG, a flagellar motor switch protein, in *L. interrogans* serovar Lai strain 56601. The second change was a single nucleotide insertion in LMANv1_2750008 (insertion of a G nucleotide in codon 234, resulting in a frameshift), which is orthologous to a gene encoding a sulfatase (la1276) in *L. interrogans* serovar Lai strain 56601.

Growth curves and osmotic stress assay. *L. interrogans* serovar Manilae strain L495 and the lb139$^-$ mutant were cultured at 30°C in EMJH to ~1×10^8 ml^{-1}. For growth rate measurements, strains were initially enumerated by the use of Petroff-Hausser counting chambers and diluted to a starting bacterial concentration of ~5×10^6 ml^{-1}. Growth was monitored daily via measurements of the optical density at 420 nm on a spectrophotometer and subsequent transformation to bacterial concentrations. Growth curves were monitored at 30°C and 37°C. For the osmotic stress assay, strains were diluted to ~1×10^7 ml^{-1} and incubated in triplicate in EMJH supplemented with NaCl at concentrations ranging from 18 to 654 mM in a total volume of 200 μl at 30°C for 72 h. After incubation, survival was measured via alamarBlue (Life Technologies) staining by addition of 20 μl alamarBlue directly to the cultures and overnight incubation at 30°C. Wells demonstrating a chromogenic shift from blue to pink were considered to contain viable bacteria.

Hamster infection and quantification of bacterial burden in organs. *Leptospira* strain L495, the lb139$^-$ mutant, and the lb139$^{-/+}$ complemented strain were enumerated via dark-field microscopy. Groups of 6 hamsters were infected via intraperitoneal (i.p.) injection of 10^6 L495 bacteria (50% infective dose [ID_{50}], <100) or 10^6 lb139$^-$ bacteria, and animals were monitored for 25 days. Blood, liver, and kidneys were collected from animals that succumbed to infection and from those that were sacrificed at day 25. The immunofluorescence imprint method was used for the detection of leptospires in kidney samples from infected hamsters (21). In a separate experiment, lb139$^-$ mutant-infected animals ($n = 4$) were sacrificed 5 days after infection, and kidneys were harvested for *Leptospira* culturing and for quantitative real-time PCR (qPCR) targeting *lipL32* (22) or *lfb1* (23), as described previously (24). In two independent experiments, groups of 6 hamsters were also challenged via the subconjunctival (CJ) route by inoculating 10^7 leptospires in 10 μl of EMJH in the conjunctiva of the left eye using a micropipette, and the animals were monitored for 21 days. For the lb139$^{-/+}$ strain, 10^6 bacteria were injected intraperitoneally into 4 hamsters, and the animals were monitored for 25 days.

The protocols for the animal experiments were prepared according to the guidelines of the Animal Care and Use Committees of Yale University

TABLE 1 Primers used for RT-PCR quantification of motility/chemotaxis genes

Open reading frame	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
rpoB	ATGGAGCGGAACGTGTAGTC	CTTCGTTCGTTCCATGTCCT
la2421	TTAGAGCGGATCCAAAACGG	ACCATGATGTGCTTCCACGA
la2422	TCAAACGGCCATCGATAGCA	ACCGATACCACTACCTTGGGA
la2423	AGTAGACGGTCAGGATGGCT	TGGTTTAGTCAGCCAGGCTC
la2425	ATCGCACTTGTCGGTGAGTT	CAATTCTGTTTCGCCGCCAA
la2426	ACAATGGACAAGCTGAGCGA	TCATCGCCTTGACGGTTTCA
la2427	CCGGGAGTGATCAACCTTCG	CCAAAGGTGGGAGGAGGTTC
la2428	GGCTCACGTTATGCTCCCTT	CCCCCTAAGTCTTCGGAGGA
la2429	TCCAGTTGGGTTCTGCGTAC	TGAGCGATCGTATGTGCTCC
lb138	GAATTCGATCACGCTCTCAA	TCTTTAGCGCTTTGTTTGGA
lb137	GTGGCATTTCGGTTCAAA	TTCTTTCGGAGGAGGTTCTG
lb136	TTGGACTTATCGGCTACAACA	TTTATTAATCGGGCTAGGAATTG

and Institut Pasteur of Paris and of New Caledonia, and these studies were approved by those committees.

RNA extraction, RNA sequencing (RNA-Seq) analyses, and reverse transcriptase PCR (RT-PCR). The *Leptospira* L495 and lb139$^-$ strains were cultured to a density of 2×10^8 ml^{-1} at 30°C with shaking. Strains were cultured in duplicate in a volume of 35 ml. Cultures were harvested via centrifugation at 3,200 × *g*, and RNA was extracted using the TRIzol (Invitrogen) method, as previously described (25).

For RNA-Seq, enriched mRNA was obtained from 7 μg of total RNA using the rRNA modified capture hybridization approach from the MicrobExpress kit (Ambion), according to the manufacturer's instructions. Enriched mRNA was then fragmented using a fragmentation kit (Ambion) and purified on RNeasy MinElute columns (Qiagen). For strand-specific high-throughput sequencing, directional cDNA libraries were prepared from enriched fragmented mRNA using a TruSeq small RNA sample preparation kit (Illumina). Fragments of cDNA of 150 bp were purified from each library, quality was confirmed on a Bioanalyzer apparatus (Agilent), and fragments of 50 bp were sequenced in single-end mode using an Illumina HiSeq2000 instrument (Illumina). Reads were cleaned from the adapter sequences and from sequences of low quality using an in-house program. Only sequences with a minimum length of 30 nucleotides were considered for further analysis. The Bowtie program, version 0.12.7 (26), was used to align the reads to the *Leptospira* genome (*L. interrogans* serovar Manilae strain L495 chromosome LMANv1_ LMANv1 [https://www.genoscope.cns.fr/agc/microscope/about/collabprojects.php?P_id=15]). Reads were counted using the option intersection nonempty from the htseq_count program (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html).

Statistical analyses were performed with the R (http://www.R-project.org) and Bioconductor (27) programs. Genes having a null read count in all the samples were excluded from further analysis. The DESeq package (version 1.8.3) was then used for normalization and differential analysis with default parameters (28). We used the nbinomTest function to compare L495 and lb139$^-$ mutant RNA samples. Genes were considered differentially expressed when the adjusted *P* value (according to the Benjamini and Hochberg procedure [29]) was below 0.05. A summary of the RNA sequencing data is shown in Table S1 in the supplemental material.

The RNA samples described above were used for quantitative RT-PCR (qRT-PCR) to confirm the RNA-Seq data. Synthesis of cDNA and qRT-PCR were performed as previously described (25) but with the following modification. The normalizing gene used in the present study was *rpoB* (la3420), and the primers used for qRT-PCR experiments are listed in Table 1.

For qRT-PCR of lb139-lb136 in the wild-type serovar Manilae L495, lb139$^-$, and lb139$^{-/+}$ strains, the strains were cultured at 30°C in triplicate to 1×10^8 ml^{-1} in 10-ml culture volumes and RNA was extracted as described above. First-strand cDNA synthesis was carried out using an iScript cDNA synthesis kit (Bio-Rad), and qRT-PCR was performed as described above.

To delineate whether the lb139-lb136 genes were cotranscribed as a polycistronic mRNA, primers were designed to amplify cDNA between lb139 and lb138, lb138 and lb137, and lb137 and lb136. The primers used for these experiments were lb139-lb138F (5′-GAAGACCACCGAACCATTTG; annealing to bp 1329 to 1351 in lb139 [1,494 bp]), lb139-lb138R (5′-AGGAATCCTACAATTGCGATG; annealing to bp 17 to 38 in lb138 [1,458 bp]), lb138-lb137F (5′-TCGATTCGATAGGTCTTATGAAAA; annealing to bp 1271 to 1294 in lb138 [1,458 bp]), lb138-lb137R (5′-ATCCGATGTTCCTTCGGTTT; annealing to bp 110 to 129 in lb137 [999 bp]), lb137-lb136F (5′-CACTTTTCCGGCTACATTCTC; annealing to bp 888 to 908 in lb137 [999 bp]), and lb137-lb136R (5′-GTACTCTCGCCCCTTCATCA; annealing to bp 162 to 181 in lb136 [399 bp]). Real-time PCR was performed as described above using genomic DNA or cDNA from wild-type strain L495. Assays with negative controls lacking reverse transcriptase during cDNA synthesis and no template in RT-PCRs were performed to ensure that cDNA samples were free of genomic DNA and that primers did not self-amplify, respectively.

Chemotaxis/motility assays. *Leptospira* strains were tested for motility using soft agar assays as previously described (30). The assays were repeated 4 times with similar results each time. To assess the motility of strains in liquid medium, we performed video microscopy using a BX53 Olympus microscope equipped with an Hamamatsu 2.8 Orca flash camera (Olympus). Wild-type L495 and the lb139$^-$ mutant were suspended in 1% methylcellulose (viscosity, 15 cP) and were observed via microscopy at a ×20 magnification. For each strain, 10 independent 2-min videos were captured for trajectory analysis. Using ImageJ software and Matlab scripts adapted from BACTRACK software (http://www.rowland.harvard.edu/labs/bacteria/software/index.php), we analyzed the trajectories for a total of 100 individual bacteria of each strain in two independent experiments. Statistical analysis was performed via the chi-square test.

Nucleotide sequence accession numbers. The genome sequence of *L. interrogans* serovar Manilae mutant M77 (lb139$^-$) is available in GenBank. This Whole-Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under accession number JFZO00000000. The version described in this paper is deposited under accession number JFZO01000000.

RESULTS

lb139 and lb136 as potential regulatory genes. The lb139 protein (497 amino acids) in *L. interrogans* displays sequence similarity to phosphatases IcfG (identity = 27.8%; proportion of matching amino acids = 46.1% over 286 amino acids) and RsbU (identity = 28.2%; proportion of matching amino acids = 51.5% over 205 amino acids) from *Synechocystis* sp. strain PCC 6803/Kazusa and *Bacillus subtilis* strain 168, respectively. More detailed amino acid sequence analysis revealed two N-terminal transmembrane domains followed by extracellular/periplasmic loops, a HAMP domain, and a C-terminal protein phosphatase 2C (PP2C) domain. The macrodomain organization resembles that of bacterial sensor proteins. Similar analyses also indicated that the lb138 and lb137 genes, which are immediately downstream of lb139, do not share significant homology to any known proteins, whereas lb136, the gene immediately downstream of lb137, is annotated as an anti-sigma factor antagonist (Fig. 1A).

Phylogenetic analysis revealed that the lb139-lb136 locus is conserved in the genomes of pathogenic strains of *L. interrogans* serovars Lai, Copenhageni, and Manilae with (Fig. 1A), in which it displays more than 99% identity. A locus sharing 21% to 38% identity exists in the saprophyte *Leptospira biflexa* (with the exception of the anti-sigma factor antagonist, which is not encoded in the same locus), but not in the intermediate species *Leptospira*

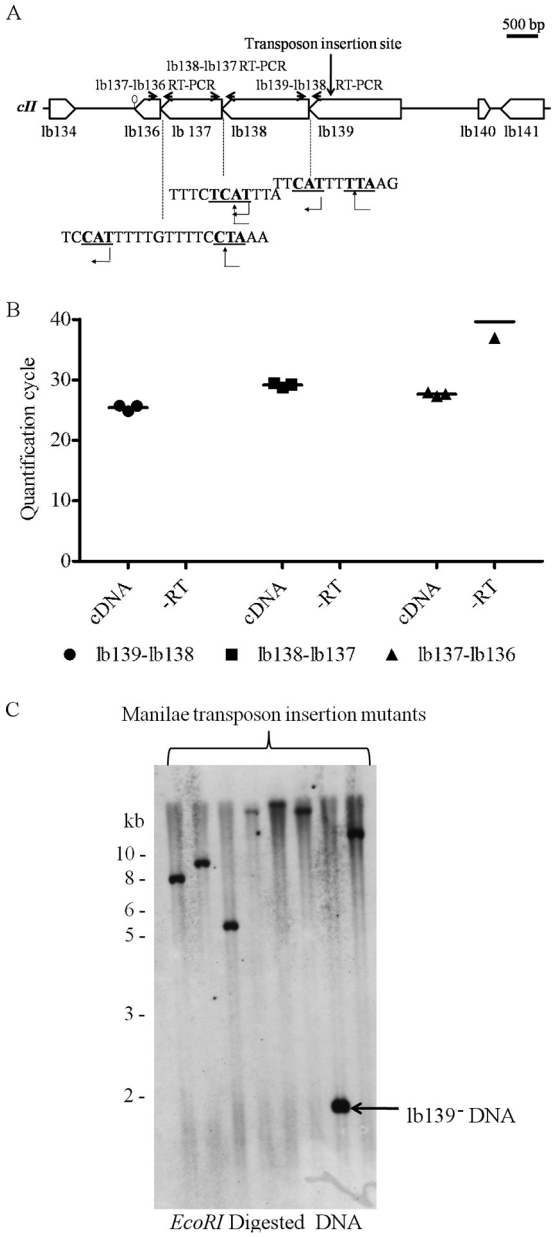

FIG 1 Single transposon insertion event in the lb139⁻ mutant and cotranscription of genes in the lb139-lb136 locus. (A) The Himar1 transposon insertion site in lb139 is indicated. Intergenic sequences displaying stop and start codons between adjacent genes are specified. A putative transcriptional terminator (stem-loop structure) is shown at the termination of lb136. Arrows between genes display regions of RT-PCR amplification used to determine whether genes were cotranscribed. The nomenclature used for genes reflects that for *L. interrogans* serovar Lai strain 56601. (B) Real-time PCR was performed on cDNA or on reaction mixtures where reverse transcriptase was omitted (−RT) during cDNA synthesis. (C) Southern blot displaying single insertion events in various *L. interrogans* serovar Manilae strain L495 transposon insertion mutants, including the lb139⁻ mutant.

licerasiae. Real-time PCR analysis on cDNA from wild-type strain L495 suggested that the lb139-lb136 locus may be transcribed as a polycistronic mRNA (Fig. 1A and B).

Transposon insertion into lb139 attenuates virulence in hamsters. Southern blot analysis suggested a single insertion event in the lb139⁻ mutant (Fig. 1A and C). The lb139⁻ mutant displayed a growth rate similar to that of wild-type strain L495 *in vitro* at 30°C but showed an enhanced growth rate at 37°C (Fig. 2A). To determine the role of the lb139-lb136 gene cluster in the infection process, the lb139⁻ mutant was tested for virulence in the hamster infection model (Fig. 2B to D). Hamsters challenged i.p. with 10^6 lb139⁻ strain bacteria survived to 25 days without any symptoms of infection, whereas hamsters challenged with the same number of bacteria of parent strain L495 died at 4 days postinfection (Fig. 2B). Similarly, hamsters challenged CJ with 10^7 lb139⁻ mutant bacteria survived without disease manifestation, whereas all animals challenged with the parental strain died by day 12 postinfection (Fig. 2C). Leptospires were not detected in the kidney samples of lb139⁻ mutant-infected animals (i.p. challenge) at day 25 postinfection (data not shown). qPCR analysis of leptospiral DNA in the blood, liver, and kidneys of i.p. infected animals (at 5 days postchallenge) demonstrated bacterial burdens at least 3 orders of magnitude lower for the lb139⁻ mutant than for parental strain L495 (Fig. 2D). Complementation of the lb139⁻ strain with lb139 did not restore virulence in hamsters (data not shown), nor did it restore transcription of lb138-lb136 to levels comparable to those in the parental strain (Fig. S1A in the supplemental material).

Altered transcription of chemotaxis/motility and signal transduction genes in the lb139⁻ mutant. Genome sequencing of the lb139⁻ mutant identified one nonsynonymous SNP in *fliG* (la0025; codon 198 GGT to GAT [G to D]), a flagellar motor switch protein, and a single nucleotide insertion in a sulfatase-encoding gene, la1276 (insertion of a G nucleotide in codon 234, resulting in a frameshift). As neither one of the last two genes encoded regulatory proteins and because of a single insertion event in lb139, transcriptional changes in the lb139⁻ mutant warranted further investigation. To identify genes altered in transcription in the lb139⁻ strain, we performed whole-transcriptome analyses via RNA-Seq. Macroanalysis revealed the downregulation of 115 genes and the upregulation of 28 genes (Tables 2 and 3). The cutoff value for genes to be considered deregulated was ±2-fold. Interestingly, the levels of transcripts of lb138, lb137, and lb136 were decreased by factors of −10.6, −7.2, and −4.8, respectively. Real-time PCR was performed on selected genes, including lb138, lb137, and lb136, to validate the RNA-Seq data.

To determine if a bias existed toward genes involved in similar biological functions, deregulated genes were categorized into COG categories and the resulting frequencies were compared to the genome-wide predicted frequencies (Fig. 3). This approach identified an approximately 4-fold higher frequency of genes involved in signal transduction (T) and of genes involved in chemotaxis and motility (N) in the downregulated category (Fig. 3).

We confirmed the downregulation of the operon la2421-la2429 (all genes encompassing la2421 to la2429, with the exception of la2424, which we did not test in RT-PCR experiments), which encodes orthologs of chemotaxis proteins CheA, CheW, CheD, CheB, CheY, and MCP. In other bacteria, CheW is a linker between the membrane sensor MCP and the kinase CheA which upon autophosphorylation transfers the phosphoryl group to either CheY or CheB (31). Upon phosphorylation, the last two proteins are activated, resulting in altered MCP methylation and interaction with the flagellar switch, which in turn regulates the flagellar motor rotation (31).

The bias toward downregulated genes coding for proteins in-

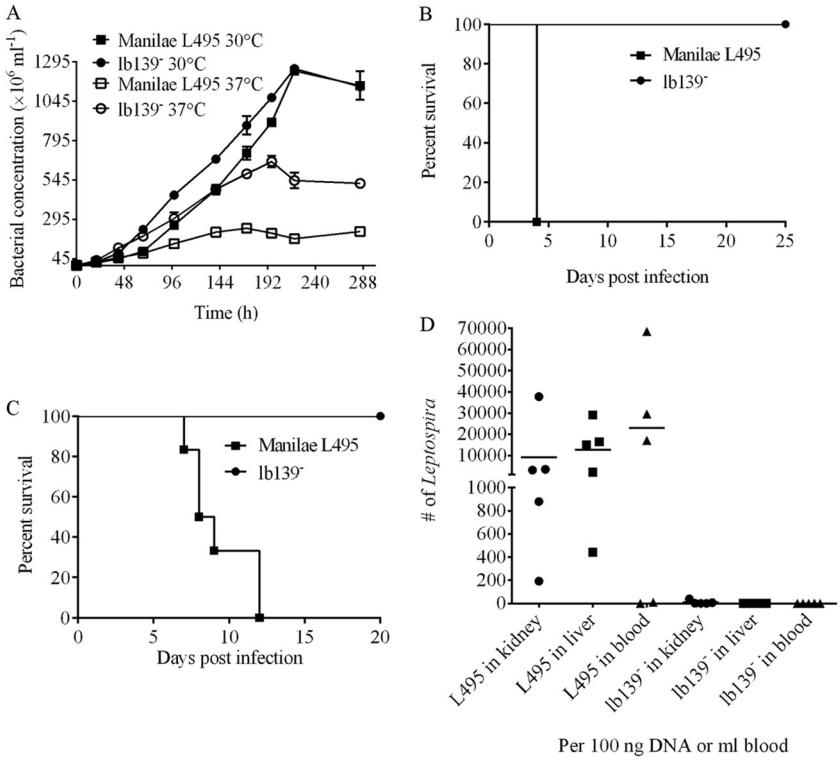

FIG 2 The lb139$^-$ strain is attenuated in the hamster infection model. (A) Growth curves demonstrating similar growth rates at 30°C and enhanced growth at 37°C for the lb139$^-$ mutant. Data are representative of those from 2 independent experiments. For virulence experiments, hamsters were challenged intraperitoneally (10^6 bacteria per animal) (B) or subconjunctivally (10^7 bacteria per animal) (C) with parental strain L495 or the lb139$^-$ mutant strain. Animals challenged intraperitoneally were monitored for 25 days, and those challenged subconjunctivally were monitored for 21 days. (D) In a separate experiment, groups of 5 hamsters were challenged intraperitoneally with the lb139$^-$ strain and sacrificed at 5 days postinfection. Blood, liver, and kidneys were collected and used for DNA extraction for detection of *Leptospira* via real-time PCR. For strain L495, blood, liver, and kidneys were collected postmortem (day 5 postinfection) and analyzed as described above.

volved in motility prompted us to investigate whether the lb139$^-$ strain displayed an altered chemotactic/motility phenotype. We first confirmed the downregulation of chemotaxis and motility transcripts via quantitative RT-PCR (Fig. 4A and B). Soft agar assays (30) demonstrated a smaller diameter in the growth ring for the lb139$^-$ strain (Fig. 4C), and tracking assays, performed via video microscopy, revealed that the average speed of the lb139$^-$ mutant strain was significantly reduced compared to that of the wild-type strain (3.72 ± 0.10 μm/s versus 6.7 ± 0.39 μm/s; $P < 0.001$).

Deregulation of numerous genes encoding extracellular proteins in the lb139$^-$ mutant. The majority (50 to 60%) of deregulated genes encoded proteins of unknown function (Fig. 3). Interestingly, of the 70 downregulated gene products of unknown function, 20 were predicted to be localized to the extracellular space, a statistically significant overrepresentation (Fisher's exact test, $P < 0.0136$) compared to the genome-wide expected frequencies (Table 4). Another notable finding was that among the downregulated genes of unknown function, 13 genes were previously demonstrated to be upregulated at physiological osmolarity (8). It follows that of the 25 genes that displayed the highest upregulation in response to physiological osmolarity (8), 16 displayed downregulation in the lb139$^-$ strain (Table 2). Notable genes included the sphingomyelinase C precursor (Sph2), the LigB lipoprotein (LigB), and conserved hypothetical lipoprotein LipL53. We tested the susceptibility of the lb139$^-$ mutant to osmotic stress and found that it demonstrated sensitivity comparable to that of wild-type L495 (see Fig. S1B in the supplemental material). However, the lb139$^-$ mutant demonstrated somewhat better growth under the tested conditions, as indicated by more intense chromogenic shifts (see Fig. S1B in the supplemental material), in accord with the robust growth rates observed *in vitro* (Fig. 2A).

DISCUSSION

The ability of pathogenic bacteria, especially those transmitted from the environment to the host, to adapt to the host milieu is crucial for virulence. Sensing changes in the external locale and the subsequent transcriptional response are initial processes of bacterial adaptation to a new environment. Thus, proteins and genes involved in the aforementioned activities serve as interesting candidates for the study of bacteria during host infection. It follows that numerous response regulators and sigma factors have been implicated in bacterial virulence in previous studies (32–34), but similar virulence regulation has not previously been determined in *Leptospira*. Only one study has addressed the role of PerR as a regulator of leptospiral genes involved in stress resistance (5); however, the same study also demonstrated that inactivation of PerR did not reduce virulence in the hamster infection model, when animals were challenged through infection of the peritoneal cavity (5). The results from the present study suggest that the genetic locus encompassing the lb139-lb136 genes may be re-

TABLE 2 Downregulated genes in the lb139$^-$ strain

Locus tag	Fold change	Description of gene product	COG[a]	Predicted location[b]
lb138	−10.6	Hypothetical protein	R/S/−	UNK
la3867[c]	−10	Putative lipoprotein	R/S/−	EX[d]
lb216	−9.5	Putative lipoprotein	R/S/−	EX[d]
lb217	−8.8	Hypothetical protein	O	UNK
lb137	−7.2	Hypothetical protein	R/S/−	IM
la3834[c]	−6	Conserved hypothetical protein	R/S/−	EX[d]
lb136	−4.8	Putative lipoprotein	T	UNK
lb225	−4.7	Conserved hypothetical protein	R/S/−	EX[d]
la1569	−4.6	Putative lipoprotein	R/S/−	EX[d]
la3829	−4.2	Hypothetical protein	R/S/−	UNK
la2473[c]	−4.1	Transcriptional regulatory protein	T	CYT
lb218	−4	Hypothetical protein	R/S/−	IM
la1183	−3.8	Hypothetical protein	R/S/−	CYT
la3881	−3.8	Outer membrane protein with integrin-like repeat domains	R/S/−	EX[d]
la1567[c]	−3.7	Putative lipoprotein	R/S/−	EX[d]
la0363	−3.6	Hypothetical protein	R/S/−	CYT
la0424	−3.5	Hypothetical protein	R/S/−	UNK
la2424	−3.5	Anti-sigma factor antagonist	R/S/−	UNK
lb093	−3.4	Long-chain fatty acid-coenzyme A ligase	I	CYT
la3647	−3.3	Hypothetical protein	R/S/−	UNK
la3377	−3.3	Hypothetical protein	R/S/−	CYT
la3974	−3.2	Exonuclease	R/S/−	UNK
la4127[c]	−3.2	Two-component system sensor histidine kinase	R/S/−	IM
la1430[c]	−3.2	3-Oxoacyl-(acyl carrier protein) synthase	I, Q	CYT
la2423	−3.2	CheY	T	CYT
la0426	−3.2	Hypothetical protein	R/S/−	EX[d]
la4282	−3	Hypothetical protein	R/S/−	CYT
la1429[c]	−3	Hypothetical protein	R/S/−	UNK
la0163[c]	−2.9	Hypothetical protein	R/S/−	CYT
la2863	−2.9	Rhodanese-like thiosulfate sulfur transferase	P	UNK
la0502	−2.8	Fatty acid desaturase	I	IM
la2720	−2.8	Hypothetical protein	R/S/−	UNK
la0286	−2.7	Hypothetical protein	N	CYT
la3798[c]	−2.7	Hypothetical protein	R/S/−	CYT
la0423	−2.7	Conserved hypothetical protein	R/S/−	EX[d]
la4126	−2.7	Sensory transduction histidine kinase	T	IM
la3145[c]	−2.7	Hypothetical protein	R/S/−	UNK
la4052	−2.7	Short-chain dehydrogenase	I, Q	CYT
la3662	−2.7	Two-component system hybrid sensor regulator	T	IM
la2020[c]	−2.7	Hypothetical protein	R/S/−	UNK
la2425	−2.7	Chemotaxis protein histidine kinase	N, T	CYT
la0587	−2.6	Lactonizing lipase	V	EX[d]
la1691[c]	−2.6	Putative lipoprotein	R/S/−	EX[d]
la0598	−2.6	Transcription regulator	R/S/−	UNK
la1743	−2.6	CheR1	N, T	CYT
la3778[c]	−2.5	LigB	R/S/−	EX[d]
la0337	−2.5	Hypothetical protein	R/S/−	UNK
la1400[c]	−2.5	Hypothetical protein	R/S/−	UNK
la2421	−2.5	Histidine kinase sensor protein	T	IM
la2422	−2.5	Histidine kinase	T	UNK
la1186[c]	−2.5	Two-component response regulator	T	CYT
la1499	−2.5	Integrin-like repeat domains	R/S/−	EX[d]
la3661	−2.4	Hydrolase	R/S/−	UNK
la3077	−2.4	Hypothetical protein	J	UNK
la2811	−2.4	Conserved hypothetical protein	R/S/−	EX[d]
la3831	−2.4	Outer membrane protein with alpha-integrin-like repeat domains	R/S/−	EX[d]
la3731	−2.4	Hypothetical protein	R/S/−	EX[d]
la2973	−2.4	Hypothetical protein	R/S/−	UNK
la2803	−2.4	Tetratricopeptide repeat-containing protein	R/S/−	UNK
la2428	−2.3	Probable chemoreceptor glutamine deamidase	N, T	UNK
la2427	−2.3	CheW	N, T	CYT

(Continued on following page)

TABLE 2 (Continued)

Locus tag	Fold change	Description of gene product	COG[a]	Predicted location[b]
la0802	−2.3	PilF tetratricopeptide repeat-containing protein	R/S/−	UNK
la3870	−2.3	Conserved hypothetical protein	R/S/−	OM[d]
la3779[c]	−2.3	Hypothetical protein	R/S/−	CYT
la2426	−2.3	Methyl-accepting chemotaxis protein	N, T, O, L	UNK
la3797	−2.3	Hypothetical protein	R/S/−	UNK
la1029[c]	−2.3	Sphingomyelinase C precursor (Sph2)/hemolysin	R/S/−	EX[d]
la0535	−2.3	Hypothetical protein	R/S/−	CYT
la0425	−2.3	Hypothetical protein	R/S/−	IM
la1276	−2.3	Sulfatase	P, M	IM
la0676	−2.2	Methyl-accepting chemotaxis protein	N, T	CYT
la0678	−2.2	Methyl-accepting chemotaxis protein	N, T	IM
la2926	−2.2	Sensory box/GGDEF family protein	T	CYT
la2813	−2.2	Methyl-accepting chemotaxis protein	N, T	IM
la0156	−2.2	Hypothetical protein	T	UNK
la3681	−2.2	Hypothetical protein	R/S/−	UNK
la1744	−2.2	CheB2	N, T	CYT
la2545	−2.2	Hypothetical protein	R/S/−	UNK
la2975	−2.2	Hypothetical protein	R/S/−	UNK
lb092	−2.1	Hypothetical protein	R/S/−	IM
la3049	−2.1	Hypothetical protein	R/S/−	IM
la2827	−2.1	Signal transduction protein	T	CYT
la0107[e]	−2.1	Hypothetical protein	R/S/−	UNK
la3839	−2.1	Phospholipid binding protein	R/S/−	PER
la1483	−2.1	Response regulator	T	UNK
la2429	−2.1	CheB3	N, T	CYT
la1423[c]	−2.1	3-Oxoacyl-(acyl carrier protein) synthase (fragment)	I	UNK
la0364	−2.1	Hypothetical protein	R/S/−	UNK
la2802	−2.1	Hypothetical protein	R/S/−	UNK
lb152	−2.1	CobP cobinamide kinase	H	UNK
la2272	−2.1	Hypothetical protein	E, G	EX[d]
la2887	−2.1	Fur (ferric uptake regulator)	P	UNK
la1859	−2.1	Catalase	R/S/−	UNK
la0142	−2.1	Hypothetical protein	R/S/−	UNK
la2200	−2.1	Amidase	V	UNK
la1422	−2.1	Serine/threonine kinase	T	UNK
la1468	−2.1	Putative lipoprotein	R/S/−	UNK
la1424[c]	−2.0	3-Oxoacid coenzyme A transferase	I, C	CYT
la3316	−2.0	Hypothetical protein	R/S/−	UNK
la2452	−2.0	Leucine-rich repeat-containing protein	R/S/−	EX[d]
la2574	−2.0	Putative methyl-accepting chemotaxis protein	N, T	IM
la3179	−2.0	Hypothetical protein	R/S/−	CYT
lb333[c]	−2.0	Response regulator	K, T	CYT
la0263	−2.0	Hypothetical protein	R/S/−	CYT
la3235	−2.0	Adenylate/guanylate cyclase	R/S/−	UNK
la1552	−2.0	Histidine kinase sensor protein	T	IM
la3333	−2.0	Conserved hypothetical protein	R/S/−	EX[d]
la1404	−2.0	Putative outer membrane protein	R/S/−	OM[d]
la3854	−2.0	Hypothetical protein	R/S/−	UNK
la1745	−2.0	Histidine kinase and response regulator hybrid protein	N, T	IM
la2974	−2.0	Methylamine utilization protein Maug	P	PER
la1467	−2.0	Outer membrane protein	R/S/−	UNK
lb149	−2.0	PmgA phosphoglycerate mutase	G	UNK
la2796	−2.0	Hypothetical protein	R/S/−	OM
la3793	−2.0	Putative hemolysin	R/S/−	CYT

[a] The COG categories are as follows: R, general function prediction only; S, function unknown; −, not in COG; O, posttranslational modification, protein turnover, chaperones; T, signal transduction mechanisms; I, lipid transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism; P, inorganic ion transport and metabolism; N, cell motility; V, defense mechanisms; L, replication, recombination, and repair; M, cell wall/membrane/envelope biogenesis; H, coenzyme transport and metabolism; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; C, energy production and conversion; K, transcription.

[b] CYT, cytoplasmic; IM, cytoplasmic membrane; PER, periplasmic space; UNK, subcellular localization could not be predicted. Annotations of CYT, IM, PER, and UNK were based on MicroScope, which utilizes PSORTb prediction software.

[c] Genes demonstrated to be upregulated at least 2-fold in *Leptospira* upon exposure to physiological osmolarity (8).

[d] Annotations for subcellular localization to the outer membrane (OM) and extracellular space (EX) are based on a previous study (49).

[e] Genes demonstrated to be downregulated at least 2-fold in *Leptospira* upon exposure to physiological osmolarity (8).

TABLE 3 Upregulated genes in the lb139$^-$ strain

Locus tag	Fold change	Description of gene product	COG[a]	Predicted location[b]
la0903	3.4	Methylase	V	UNK
la2565	3.2	Thioesterase	R/S/−	UNK
la0146	2.7	Transcriptional regulator	K, T, O	CYT
la0352	2.7	Hypothetical protein	R/S/−	IM
la0905	2.3	Hypothetical protein	R/S/−	UNK
la1999	2.3	Hypothetical protein	R/S/−	CYT
la1188	2.3	Hypothetical protein	R/S/−	UNK
la2084	2.3	Hypothetical protein	E, G, P	IM
la1476	2.2	Dehalogenase-like hydrolase	R/S/−	IM
la4049	2.1	DEAD/DEAH box helicase	L, J, K	CYT
la3965	2.1	Na^+-driven multidrug efflux pump	V	UNK
la0568	2.1	Fatty acid transport protein	R/S/−	OM[c]
la0898	2.1	Hypothetical protein	R/S/−	UNK
la0964	2.1	ATP-dependent DNA helicase RecQ	L, K	CYT
la2035	2.1	Hypothetical protein	R/S/−	CYT
la0906	2.1	Hypothetical protein	R/S/−	UNK
la1464	2.1	UDP-*N*-acetylmuramoyl-tripeptide–D-alanyl-D-alanine ligase	M	CYT
la3173	2.1	ATPase component of ABC transporter	V, E, P	IM
la3392	2.1	Hypothetical protein	R/S/−	UNK
la3064	2.0	Putative lipoprotein	R/S/−	EX[c]
la4167	2.0	CoaD phosphopantetheine adenyl transferase	H	CYT
la0693	2.0	Aspartokinase LysC	E	UNK
la2988	2.0	PyrE	F	CYT
lb074	2.0	Mcm2	I	UNK
la4026	2.0	Hypothetical protein	R/S/−	OM[c]
la3139	2.0	Cytochrome *c* peroxidase	P	PER
la2987	2.0	Hypothetical protein	R/S/−	UNK

[a] The COG categories are as follows: V, defense mechanisms; R, general function prediction only; S, function unknown; −, not in COG; K, transcription; T, signal transduction mechanisms; O, posttranslational modification, protein turnover, chaperones; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; P, inorganic ion transport and metabolism; L, replication, recombination, and repair; J, translation, ribosomal structure, and biogenesis; M, cell wall/membrane/envelope biogenesis; H, coenzyme transport and metabolism; F, nucleotide transport and metabolism; I, lipid transport and metabolism.

[b] CYT, cytoplasmic; IM, cytoplasmic membrane; PER, periplasmic space; UNK, subcellular localization could not be predicted. Annotations of CYT, IM, PER, and UNK were based on MicroScope, which utilizes PSORTb prediction software.

[c] Annotations for subcellular localization to the outer membrane (OM) and extracellular space (EX) are based on a previous study (49).

quired for the regulation of genes whose products are necessary for leptospiral virulence.

The results of the RNA-Seq experiments support the hypothesis that the protein products of lb139-lb136 contain regulatory functions. These experiments demonstrated decreased transcription of the lb138-lb136 genes but not lb139, even though the insertion site was within lb139. The insertion site in lb139 resides near the 3′ end of the gene, which suggested that lb139 was transcribed, and it was thus detected in RNA-Seq and RT-PCR experiments. It should be highlighted that the protein product of the resulting lb139 would be truncated and nonfunctional, since the insertion occurs within the phosphatase domain. Furthermore, we conclude that insertion into lb139 leads to a 4.8- to 10.6-fold decrease in the transcription of the lb136-lb138 genes. We have tested via RT-PCR the possibility that the lb139-lb136 genes are cotranscribed as an operon in the wild-type strain, and RT-PCR demonstrated that the transcription of downstream genes commences within upstream genes. It follows that the decreased transcription of the lb138-lb136 genes was likely caused by a transposon insertion into lb139, as complementation with lb139 did not restore the transcription of these genes. It is reasonable to conclude that decreased expression of these genes would dampen their activity, which is supported by the observation that a total of 143 genes were deregulated at least 2-fold in the lb139$^-$ mutant.

Complementation with lb139 did not restore transcription to levels comparable to those for the wild type, nor did we observe the restoration of virulence and/or motility, which suggested that lb139 alone is not enough for the regulation of transcription, virulence, and motility. Furthermore, Southern blot experiments indicated a single insertion event, ruling out the possibility of other events of insertions into regulatory genes. However, genome sequencing revealed two additional changes, a single nonsynonymous mutation (confirmed by PCR and subsequent sequencing) in a flagellar motor switch protein-encoding gene (*fliG* [la0025] in serovar Lai strain 56601) and a single nucleotide insertion resulting in a frameshift in a gene encoding a protein of unknown function orthologous to a sulfatase (la1276) in serovar Lai strain 56601. It has previously been demonstrated that inactivation of la0025 does not alter *Leptospira* motility (35), and thus, the motility defect in the lb139$^-$ mutant could not be attributed to the SNP in la0025. Additionally, we do not believe that the SNP in the *fliG* gene and inactivation of la1276 could account for the extensive deregulation of 143 transcripts in the lb139$^-$ mutant, as neither one of these genes encodes regulatory proteins.

Among the downregulated genes, those involved in signal transduction and motility were overrepresented. Downregulation of 27 signal transduction genes suggested that the lb139-lb136 genes may be involved in a complex network of multiple regulatory genes acting in concert to regulate the leptospiral transcriptional response to a new environment. Thus, we do not suggest

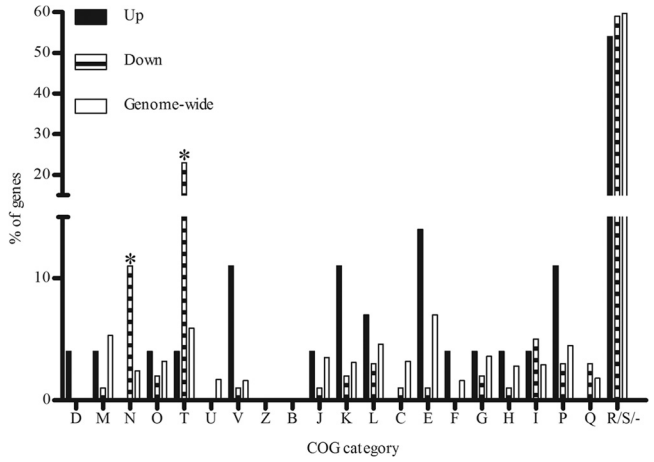

FIG 3 The transcriptome of the lb139$^-$ strain displays an overrepresentation in the frequency of genes downregulated in COG categories motility/chemotaxis and signal transduction. The COG categories for serovar Manilae strain L495, as annotated in MicroScope (http://www.genoscope.cns.fr/agc/microscope/home/index.php), are abbreviated as follows, and the predicted percentages for genes in each category are given in parentheses: D, cell cycle control, cell division, chromosome partitioning (0.9%); M, cell wall/membrane/envelope biogenesis (5.3%); N, cell motility (2.4142%); O, posttranslational modification, protein turnover, chaperones (3.2%); T, signal transduction mechanisms (5.9%); U, intracellular trafficking, secretion, and vesicular transport (1.7%); V, defense mechanisms (1.6%); Z, cytoskeleton (0.06%); B, chromatin structure and dynamics (0.04%); J, translation, ribosomal structure, and biogenesis (3.5%); K, transcription (3.1%); L, replication, recombination, and repair (4.6%); C, energy production and conversion (3.2%); E, amino acid transport and metabolism (7.0%); F, nucleotide transport and metabolism (1.6%); G, carbohydrate transport and metabolism (3.6%); H, coenzyme transport and metabolism (2.8%); I, lipid transport and metabolism (2.9%); P, inorganic ion transport and metabolism (4.5%); Q, secondary metabolite biosynthesis, transport, and catabolism (1.8%); R, general function prediction only (11.0%); S, function unknown (5.0%); −, not in COG (24.2858%). *, genes involved in motility and signal transduction were significantly (Fisher's exact test, $P < 0.0385$) overrepresented (~4-fold higher than expected) in the downregulated category.

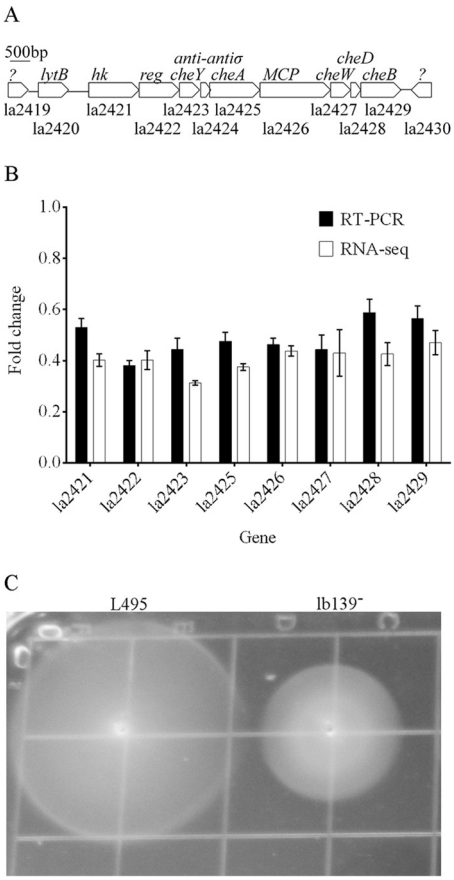

FIG 4 The lb139$^-$ strain displays reduced transcription of a chemotaxis genetic locus and exhibits reduced motility. Decreased transcription of a chemotaxis operon was observed in the lb139$^-$ strain via RNA-Seq. Select genes in this operon were quantified via RT-PCR to validate the RNA-Seq quantification data. The lb139$^-$ strain was further characterized phenotypically to determine the effect of the observed downregulation of the aforementioned genes on motility. (A) A genetic locus encoding chemotaxis genes; (B) quantification of select genes in the above-described genetic locus via RT-PCR and RNA-Seq; (C) phenotypic comparison of strain L495 and the lb139$^-$ mutant on soft agar medium. The soft agar assays were performed 4 times.

that the lb139-lb136 genes directly regulate all the genes that we observed to be deregulated; rather, we conclude that the lb139-lb136 genetic locus is crucial for mediating leptospiral virulence, either directly or indirectly. Downregulation of motility genes and the resulting phenotypic defect in motility suggested that the lb139-lb136 genetic locus is required for full leptospiral motility. It should be highlighted that leptospiral motility is required for virulence (30, 36), and thus, the reduced motility in the lb139$^-$ mutant may have partially contributed to the observed virulence attenuation.

We did not detect deregulation of all genes involved in leptospiral virulence. For example, virulence-associated genes such as *ligA*, *lipL32*, *lfhA*, *loa22*, *hbpA*, *lruA*, *lruB*, *hemO*, and *htpG* and lipopolysaccharide biosynthesis genes were not altered in expression, suggesting that the lb139-lb136 locus is not involved in the regulation of all virulence-associated genes. However, we observed downregulation of a previously described virulence-associated gene, *katE* (25), and of ~2/3 of the total number of leptospiral genes demonstrated to be upregulated in response to physiological osmolarity (8), indicating that virulence attenuation in the lb139$^-$ mutant is perhaps more complex than reduced motility alone.

Physiological osmolarity serves as a cue for *Leptospira* to both initiate expression of genes (8) and release the LigB and Sph2 proteins into the extracellular space (37). Interestingly, previous studies have also demonstrated LigB- and Sph2-mediated binding of host homeostatic proteins (38, 39), suggesting a role for these proteins in leptospiral attachment to host cells. In addition to

TABLE 4 Overrepresentation of genes encoding predicted extracellular proteins in the downregulated category in the lb139$^-$ strain

Localization[c]	% genes overrepresented (no. of genes overrepresented/total no. of genes)		
	Predicted[b]	Downregulated	Upregulated
EX	2.4 (114/4,722)	17.4[a] (20/115[a])	3.6 (1/28)
OM	1.3 (63/4,722)	2.6 (3/115)	7.1 (2/28)
CYT	17.0 (803/4,722)	20.1 (24/115)	28.6 (8/28)

[a] Fisher's exact test, $P < 0.0136$.
[b] Annotations for subcellular localization are extrapolated for serovar Manilae on the basis of a previous study in serovar Lai (49).
[c] CYT, cytoplasmic; OM, outer membrane; EX, extracellular space.

altered transcription of genes in the aforementioned biological processes, 20 genes with predicted extracellular protein products were also downregulated, including three protein products with integrin domains, LigB, and sphingomyelinase Sph2. While individually these targets have yet to be characterized as virulence proteins, codownregulation in the lb139$^-$ mutant could have the net effect of reducing bacterial fitness *in vivo*. The roles of extracellular proteins in attachment to host factors and dissemination and their requirement for the early stages of the infection process have been demonstrated in numerous other bacteria, and a similar role is likely played in leptospiral pathogenesis. This hypothesis is in accord with our observed attenuation of the lb139$^-$ mutant when hamsters were challenged via the subconjunctival route, which would necessitate attachment, traversal, and dissemination to and from host barriers for *Leptospira* to reach target organs.

Like virulence in other bacteria, leptospiral virulence is complex because of the number of mechanisms utilized to establish infection within the host. Previous studies have identified a number of biological processes that appear to be required for leptospiral virulence. These include genes involved in motility (30, 36), heme utilization (18), lipopolysaccharide biosynthesis (40), stress resistance (25, 41, 42), and the host-pathogen interaction (43) and genes that are involved in a manner that has not yet been determined (44, 45). However, previous research has also negated the requirement of putative virulence factors LipL32 (46), LipL41 (47), and LigB (48) in the animal infection model. Taken together these studies suggest plasticity and redundancy in leptospiral genomes, where gene products can mimic the function of other genes in the event of a loss of function, thereby safeguarding survival of the bacteria. In accordance with the complexity of bacterial gene regulation during the infection process, regulatory genes make interesting targets for the study of virulence, as disruption of these genes often has downstream effects on the expression and/or activity of other genes. While plasticity also exists in regulatory genes if mimicry is not satisfactory, the cumulative effect of deregulation of numerous genes could be fatal for bacteria under certain environmental conditions. Genes lb139 and lb136 encode proteins similar to the PP2C phosphatase RsbU and anti-sigma factor antagonist RsbV, respectively. This chromosomal region also contains a gene (lb144) encoding an extracytoplasmic function (ECF) sigma factor. Taken together with results indicating the cotranscription of the lb139-lb136 genes, deregulation of numerous genes in the lb139$^-$ mutant, and attenuated virulence in hamster, we speculate that the protein products of these genes are involved in the regulation of the transcriptional response in a manner essential for virulence. In the present study, we demonstrate that transposon inactivation of lb139 results in the reduced transcription of numerous genes involved in specific biological processes, including genes encoding proteins required for motility and chemotaxis, proteins expressed after a shift to physiological osmolarity, and proteins required for signal transduction, and a subset of genes encoding predicted extracellular proteins. We suggest that deregulation of these biological processes combines to reduce leptospiral fitness in hamsters. The results presented here warrant further biochemical analysis of the lb139-lb136 genetic locus in order to delineate the mechanism underlying transcriptional regulation.

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