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GENETICS AND MOLECULAR BIOLOGY

The EbpA-RpoN Regulatory Pathway of the Pathogen *Leptospira interrogans* Is Essential for Survival in the Environment

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ABSTRACT *Leptospira interrogans* is the agent of leptospirosis, a reemerging zoonotic disease. It is transmitted to humans through environmental surface waters contaminated by the urine of mammals chronically infected by pathogenic strains able to survive in water for long periods. Little is known about the regulatory pathways underlying environmental sensing and host adaptation of *L. interrogans* during its enzootic cycle. This study identifies the EbpA-RpoN regulatory pathway in *L. interrogans*. In this pathway, EbpA, a σ^{54} activator and putative prokaryotic enhancer-binding protein (EBP), and the alternative sigma factor RpoN (σ^{54}) control expression of at least three genes, encoding AmtB (an ammonium transport protein) and two proteins of unknown function. Electrophoresis mobility shift assay demonstrated that recombinant RpoN and EbpA bind to the promoter region and upstream of these three identified genes, respectively. Genetic disruption of *ebpA* in *L. interrogans* serovar Manilae virtually abolished expression of the three genes, including *amtB* in two independent *ebpA* mutants. Complementation of the *ebpA* mutant restored expression of these genes. Intraperitoneal inoculation of gerbils with the *ebpA* mutant did not affect mortality. However, the *ebpA* mutant had decreased cell length *in vitro* and had a significantly lowered cell density at stationary phase when grown with L-alanine as the sole nitrogen source. Furthermore, the *ebpA* mutant has dramatically reduced long-term survival ability in water. Together, these studies identify a regulatory pathway, the EbpA-RpoN pathway, that plays an important role in the zoonotic cycle of *L. interrogans*.

IMPORTANCE Leptospirosis is a reemerging disease with global importance. However, our understanding of gene regulation of the spirochetal pathogen *Leptospira interrogans* is still in its infancy, largely due to the lack of robust tools for genetic manipulation of this spirochete. Little is known about how the pathogen achieves its long-term survival in the aquatic environment. By utilizing bioinformatic, genetic, and biochemical methods, we discovered a regulatory pathway in *L. interrogans*, the EbpA-RpoN pathway, and demonstrated that this pathway plays an important role in environmental survival of this pathogen.

KEYWORDS *Leptospira*, RpoN, leptospirosis, spirochete

Leptospires are a group of spirochetes that include both saprophytic and pathogenic species within the genus *Leptospira* (1). Pathogenic species, including *Leptospira interrogans*, are the causative agents of leptospirosis, the most widespread zoonotic infectious disease in the world. Saprophytic species, including *L. biflexa*, are found primarily in aquatic and terrestrial environments and are considered nonpathogenic. Pathogenic leptospires also can survive in the terrestrial environment for a prolonged

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period, and humans are typically infected by direct or indirect contact with soil or water that is contaminated with the urine of infected animals.

L. interrogans must adapt to both aquatic and mammalian host conditions in order to be successfully maintained in its zoonotic cycle. Some progress has been made on how *L. interrogans* alters its gene expression to cope with environmental change. Whole-genome microarrays and high-throughput RNA sequencing analyses revealed global changes in transcript levels of *L. interrogans* in response to temperature (2), osmolality (3), iron depletion (4), serum exposure (5), interaction with phagocytic cells (6), and cultivation in a host-adapted model (the dialysis membrane chambers model) (7). Understanding how *L. interrogans* senses environmental and host signals and regulates its gene expression has just begun to be explored.

Little is known about the regulation of gene expression in *L. interrogans*. It has been shown that the PerR homolog in *L. interrogans* acts as a regulator of the oxidative stress response (3, 4, 8). The KdpE sensor was shown to be an activator of the KdpABC potassium transporter (9). The LexA repressor bound to the promoter of *recA* is involved in DNA repair (10). HemR regulates the heme biosynthetic pathway (11, 12). Despite these advances, the regulatory pathways underlying environmental sensing and host adaptation of *L. interrogans* remain poorly understood, largely due to the lack of a robust system for genetic manipulation of *Leptospira*.

One common mechanism of regulating bacterial gene expression is the use of alternative sigma factors (σ). In addition to the housekeeping sigma factor σ^{70} (RpoD), *Leptospira* species have several alternative sigma factors, including σ^{54} (RpoN), σ^{F} (FliA), σ^{B}, and 5 to 11 extracytoplasmic function sigma factors (σ^{E}) (13–15). Among these alternative σ factors, σ^{54} is phylogenetically different from the other σ factors; all other alternative σ factors belong to the σ^{70} family. σ^{54} recognizes a unique $-24/-12$ promoter sequence (instead of the $-35/-10$ promoter sequence for σ^{70}). In addition, transcriptional activation from a σ^{54}-type promoter always requires a bacterial enhancer binding protein (EBP) (16, 17). A typical EBP consists of an N-terminal allosteric effector binding domain for sensing the signal, an AAA^{+} NTPase domain for σ^{54} activation, and a C-terminal helix-turn-helix domain for DNA binding. The EBP binds to the enhancer region, where it curves toward and interacts with the σ^{54} holoenzyme. Upon sensing a signal, the EBP is activated, which in turn activates σ^{54} by AAA^{+}-type ATPase activity. This activation energy promotes the closed complex of the holoenzyme and the formation of an open complex at the promoter, resulting in the transcriptional activation of σ^{54}-dependent genes.

σ^{54} has been shown to modulate many cellular processes, including nitrogen assimilation, motility, virulence, and biofilm formation in bacteria (16–18). Previous experiments have ascertained that *rpoN*, while typically associated with pathogenic adaptation to host environment, has also been shown to play a role in ecological adaptation and survival. Therefore, we attempted to characterize the role of an *rpoN* regulon in pathogenic *Leptospira* (18–20). In this study, using a combination of bioinformatic, biochemical, and genetic approaches, we report identification of the EbpA-RpoN regulatory pathway in *L. interrogans*. This pathway constitutes a σ^{54} activator (LMANv2_37011, here referred to as EbpA), σ^{54} (LMANv2_590036, RpoN), and at least three downstream targets. We found that this pathway is not essential for mammalian infection but plays an important role in *L. interrogans* adaptation to diverse environmental conditions during the enzootic cycle.

RESULTS

***In silico* prediction of RpoN (σ^{54}) regulatory network in *L. interrogans*.** Genome sequences reveal that all sequenced *Leptospira* species (including pathogenic, saprophytic, and intermediate strains) have the alternative sigma factor σ^{54}. To study the role of σ^{54} in the enzootic cycle of *L. interrogans*, we first performed *in silico* analysis of the σ^{54} network in *L. interrogans*. Since its activation always requires an activator, an EBP, which has a conserved domain for activation of σ^{54}-polymerase-dependent transcriptional initiation, we analyzed the presence of EBPs among *Leptospira* species. Our

FIG 1 Predicted RpoN regulatory network in *L. interrogans*. The *L. interrogans* genome encodes a σ^{54} alternative sigma factor and two bacterial enhancer-binding proteins (EbpA and EbpB) that activate the genes with a σ^{54}-type promoter. Both EbpA and EbpB are predicted to have three functional domains: an N-terminal domain that receives a distinct signal, a central domain that activates σ^{54}, and a C-terminal domain that binds to DNA. EbpA contains two putative GAF domains in its receiver domain, whereas EbpB contains a receiver domain (REC) conserved for all the two-component system response regulator proteins. EbpA and EbpB respond to different signals, form oligomers, and interact with RpoN (σ^{54}), and each activates a specific set of σ^{54}-dependent genes. RNP, RNA polymerase.

analyses revealed that all pathogenic *Leptospira* have two activators (here named *Leptospira* enhancer-binding proteins A and B, i.e., EbpA [LMANv2_37011, FhlA-like] [16, 18] and EbpB [LMANv2_590040, NtrC-like] [16, 18]). These results suggest that within the σ^{54} regulon there are two pathways, EbpA-RpoN (σ^{54}) and EbpB-RpoN (σ^{54}), that activate two sets of genes that utilize a σ^{54}-type promoter (Fig. 1).

Because σ^{54} recognizes a unique $-24/-12$ promoter sequence, σ^{54}-dependent genes can be predicted *in silico*. In this regard, Francke et al. performed a comparative genome analysis of over 300 genera based on the frequency representation of the σ^{54} promoter (18). Based on their results, we searched the *L. interrogans* genomes using Promscan analysis tools (http://molbiol-tools.ca/promscan/) and queried for genes containing a putative RpoN binding sequence in putative promoter regions. We identified 13 putative RpoN (σ^{54})-type promoters for 22 genes in *L. interrogans* 56601 and L495 strains (where genes shared by the same promoter were grouped) (Table 1). Twenty-one of these genes were located on chromosome I, and one gene was located on chromosome II. Five of these genes encode hypothetical proteins, and 17 genes encode proteins with assigned functions such as nitrogen assimilation, glutamine synthesis, alanine racemization, flagellar synthesis, sodium bile acid symport, and lipid A biosynthesis. Given that *L. interrogans* has two EBPs, we predicted that one set of these 22 genes is controlled by the EbpA-RpoN pathway, and the other set is governed by EbpB-RpoN.

TABLE 1 *Leptospira* genes containing a perfect RpoN binding sequence in the putative promoter regions

Gene	Function	Matching sequence
LMANv2_200027	Tetratricopeptide repeat protein, outer membrane protein	TGGCACGCTGCTTGCA
raiA (*LMANv2_200026*)	σ^{54} modulation protein, ribosomal subunit interface protein	TGGCACGCTGCTTGCA
lpxC (*LMANv2_430009*)	UDP-3-*O*-[3-hydroxymyristoyl] *N*-acetylglucosamine deacetylase	TGGCATAGAGTTTGCT
ptsA (*LMANv2_430010*)	Phosphoenolpyruvate-protein phosphotransferase	TGGCATAGAGTTTGCT
secE (*LMANv2_260002*)	Preprotein translocase subunit	TTCAAAAGCCGTGCCG
nusG (*LMANv2_260003*)	Transcription antiterminator	TTCAAAAGCCGTGCCG
rplK (*LMANv2_260004*)	50S ribosomal protein L11	TTCAAAAGCCGTGCCG
rplA (*LMANv2_260005*)	50S ribosomal protein L1	TTCAAAAGCCGTGCCG
rplJ (*LMANv2_260006*)	50S ribosomal protein L10	TTCAAAAGCCGTGCCG
rplL (*LMANv2_260007*)	50S ribosomal protein L7/L12	TTCAAAAGCCGTGCCG
LMANv2_290065	Outer membrane protein	TGCAAAATATATGCCA
glnA (*LMANv2_500019*)	Glutamine synthetase	TGGTACAATATTTGCA
amtB (*LMANv2_310003*)	Ammonia permease	TGGTACTAATCTTGCA
glnK (*LMANv2_310004*)	Nitrogen regulatory protein P-II; nitrogen assimilation regulatory protein for GlnL, GlnE, and AmtB	TGGTACTAATCTTGCA
nctP1 (*LMANv2_210009*)	Sodium bile acid symporter	AGCAAAAAGCGCGCCG
pdxH (*LMANv2_210047*)	Pyridoxamine-phosphate oxidase	AGGCAAGCGTTTTGCT
LMANv2_240139	Putative membrane protein	ATCAAGTTGTATGCCA
LMANv2_240051	HD family protein	AGCACTTTCGATGCCA
LMANv2_300019	Putative membrane protein	TGGCACTAAATTTGAT
lepB (*LMANv2_300018*)	Signal peptidase I	TGGCACTAAATTTGAT
fliO (*LMANv2_150090*)	Endoflagellar biogenesis protein	TGCAATCAGCTTGCCA
alr (*LMANv2_90055*)	Alanine racemase	TGGCATGGACTCTGCA

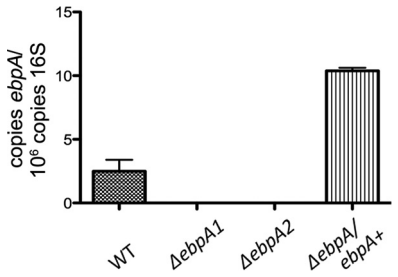

FIG 2 qRT-PCR analysis of *ebpA* expression. RNAs were isolated from wild-type (WT), the *ebpA* mutants (Δ*ebpA1* and Δ*ebpA2*), and the complemented strain (Δ*ebpA*/*ebpA*$^+$) and subjected to qRT-PCR analyses. The levels of *ebpA* expression were reported relative to the level of 16S rRNA in each sample.

Inactivation and complementation of the enhancer binding protein gene *ebpA*. To investigate the function of the σ^{54} regulon in *L. interrogans*, we initially attempted to inactivate *rpoN* (encoding σ^{54}) in *L. interrogans* strains. However, multiple attempts were unsuccessful. A random transposon mutant library in *L. interrogans* serovar Manilae strain L495 also has not yet yielded an *rpoN*-disrupted mutant (21). However, the library did yield two biologically distinct mutants with transposon insertion within the proximal region of *ebpA* (22). These biologically distinct mutants were named the *ebpA1* (transposon library ID M250) and *ebpA2* (transposon library ID M1407) mutants.

To study the function of *ebpA* and to fulfill molecular Koch's postulates, we first performed complementation for *ebpA* (referred to here as Δ*ebpA*/*ebpA*$^+$ clones) by randomly *cis* inserting a wild-type copy of *ebpA* (including its upstream native promoter) into the *ebpA1* mutant. Subsequent sequence analysis revealed that one of the Δ*ebpA*/*ebpA*$^+$ clones used here harbored an *ebpA* insertion at the distal region of *LMANv2_590053* (conserved protein of unknown function) at nucleotide 4079032. Quantitative reverse transcription-PCR (qRT-PCR) analyses demonstrated that the two *ebpA* mutants did not express *ebpA*, whereas the Δ*ebpA*/*ebpA*$^+$ complemented strain successfully restored *ebpA* expression (Fig. 2).

Deletion of *ebpA* affects cell morphology in *L. interrogans*. Qualitative examination of the *ebpA* mutant by microscopy revealed no defect in motility. However, we observed that the *ebpA* mutant appeared shorter in length than the wild-type strain. Further quantitative analyses of cell length showed that the two *ebpA* mutants were significantly shorter in cell length (7.365 μm $\pm$ 0.1782 μm [$P < 0.01$] and 7.566 μm $\pm$ 0.1631 μm [$P < 0.01$]) than the wild type (10.99 μm $\pm$ 0.3118 μm) when grown to mid- to late-logarithmic phase. Complementation of *ebpA1* restored the cell length in the Δ*ebpA*/*ebpA*$^+$ strain (10.47 μm $\pm$ 0.2129 μm) (Fig. 3).

Identification of EbpA-regulated genes by qRT-PCR. To identify genes controlled by the EbpA-RpoN pathway, initial qRT-PCR analysis by the relative fold change between the wild type and the *ebpA* mutants was performed. Among the 22 predicted RpoN-controlled genes, three—*LMANv2_31003* (*amtB*, ammonia transporter gene), *LMANv2_200027* (encoding a conserved exported protein), and *LMANv2_290065* (encoding a putative outer membrane protein)—had significantly lower expression in the *ebpA* mutant than in the wild-type strain ($P < 0.01$) (Fig. 4A). Based on this information, a second analysis was performed by comparing the absolute transcript levels of the above identified genes to copies of 16S rRNA, which is constitutively expressed under standard *in vitro* growth conditions at 30°C (23) in the wild-type, the Δ*ebpA*, and Δ*ebpA*/*ebpA*$^+$ strains. This study reconfirmed the findings presented above of significantly lower expression of *amtB*, *LMANv2_200027*, and *LMANv2_290065* in the *ebpA* mutant compared to the wild type (5- to 10-fold change) (Fig. 4B). The complemented Δ*ebpA*/*ebpA*$^+$ strain restored the expression of *amtB*, *LMANv2_200027*, and *LMANv2_290065* (Fig. 4B). These results suggest that EbpA-RpoN regulates the expression of *amtB*, *LMANv2_200027*, and *LMANv2_290065*.

RpoN and EbpA bind to the promoter region of *amtB*, *LMANv2_200027*, and *LMANv2_290065*. To demonstrate that *amtB*, *LMANv2_200027*, and *LMANv2_290065*

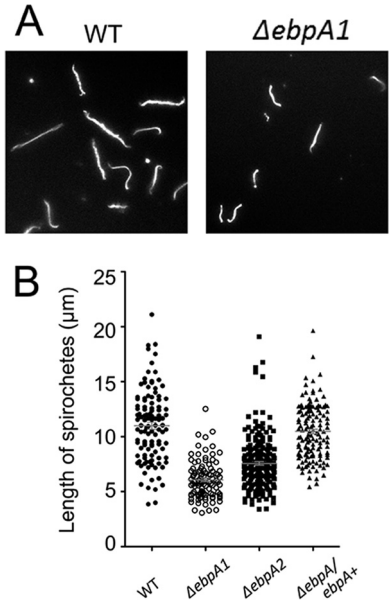

FIG 3 Cell morphology of the *ebpA* mutants and the complemented strain. (A) Morphology of wild-type and Δ*ebpA1* strains grown in EMJH medium examined under a dark-field microscope. Images were taken at a magnification of ×500. (B) Quantitation of cell length. A total of 200 randomly selected cells from each strain were measured. Wild type (WT), 10.99 ± 0.3118 μm; Δ*ebpA1* mutant, 7.365 ± 0.1782 μm; Δ*ebpA2* mutant, 7.566 ± 0.1631 μm; Δ*ebpA*/*ebpA*$^+$ strain, 10.47 ± 0.2129 μm. Comparisons: WT versus either the Δ*ebpA1* mutant or the *ebpA2* mutant, $P < 0.01$; Δ*ebpA*/*ebpA*$^+$ strain versus Δ*ebpA1* mutant, $P < 0.01$.

have an RpoN (σ^{54}) promoter, we purified recombinant RpoN of *L. interrogans* and performed an electrophoretic mobility shift assay (EMSA). A 50-bp oligonucleotide encoding the predicted −24/−12 promoter region of *amtB*, *LMANv2_200027*, and *LMANv2_290065* was end labeled with biotin and mixed with different amounts of purified recombinant RpoN in binding buffer. The results showed that *L. interrogans* RpoN bound to the predicted σ^{54} promoter of *amtB*, *LMANv2_200027*, and *LMANv2_290065* in a dose-dependent manner (Fig. 5A). The bindings were specific, since unlabeled DNA fragments could compete for binding and no binding was observed when bovine serum albumin (BSA) was used instead of RpoN. Furthermore, mutated probes (MRN027, −25TGGCA→AAAAT and −14TTGC→AATT; MRN065, −25TGGCA→AAAAT and −14TTGC→AATT; or MRN003, −25TGGTA→AAAAT and −14TTGC→AATT) in the −24/−12 region did not show binding (Fig. 5B). These data

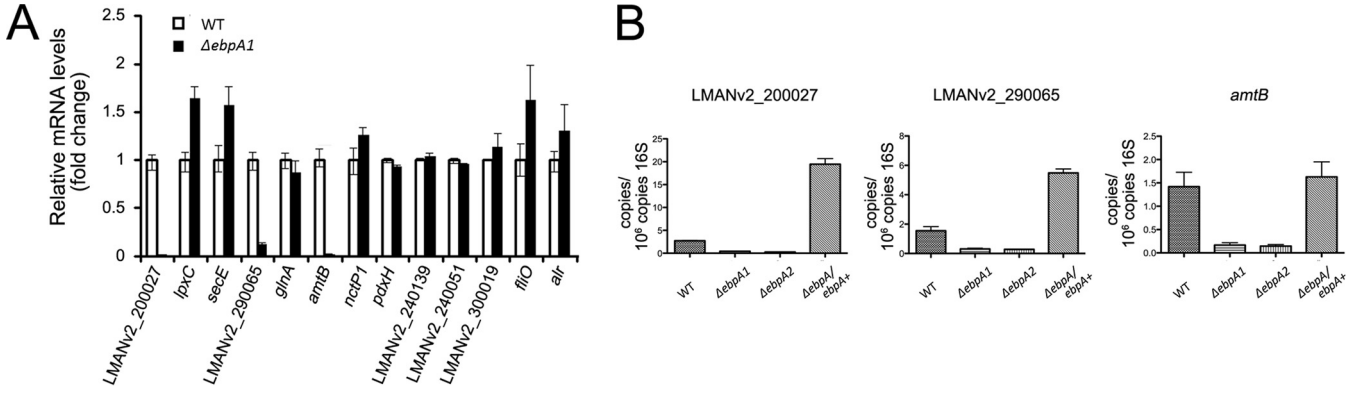

FIG 4 Identification of genes regulated by EbpA. (A) qRT-PCR assessment of expression of putative RpoN-regulated genes. (B) qRT-PCR analyses to confirm EbpA-dependent expression of *LMANv2_200027*, *LMANv2_290065*, and *LMANv2_310003/amtB*. For the expression of all three genes: wild type (WT) versus Δ*ebpA1* or Δ*ebpA2* mutant, $P < 0.01$; Δ*ebpA*/*ebpA*$^+$ strain versus Δ*ebpA1* mutant, $P < 0.01$; WT versus Δ*ebpA*/*ebpA*$^+$ strain, $P < 0.01$.

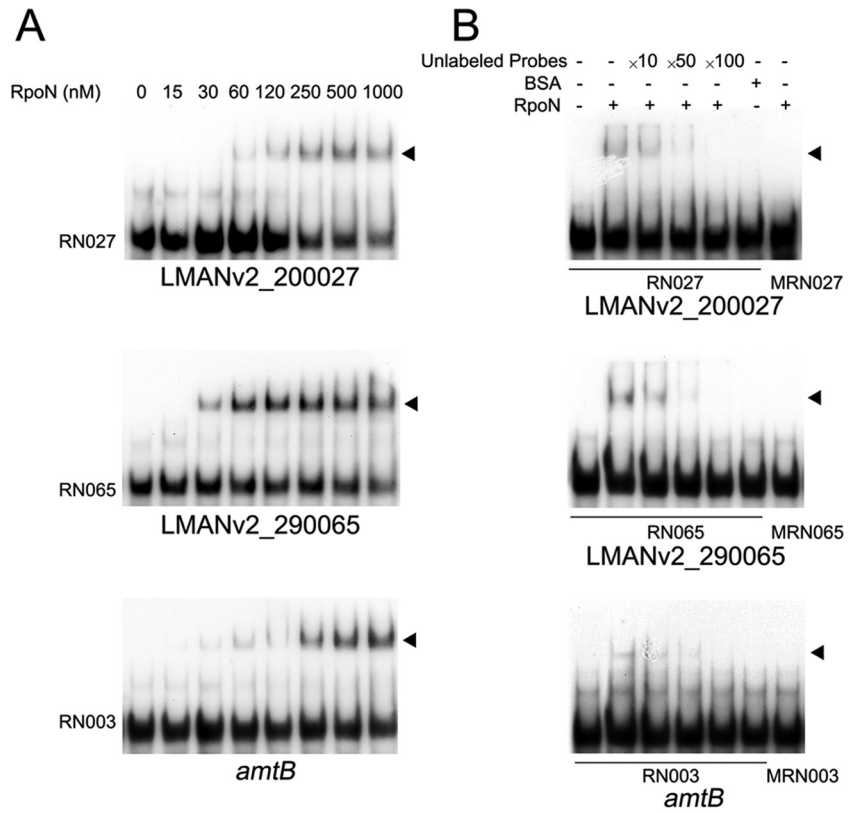

FIG 5 EMSA analysis for identification of RpoN (σ^{54})-type promoter. (A) RpoN binding to the promoter regions of *LMANv2_200027*, *LMANv2_290065*, or *amtB*. Various concentrations of rRpoN (labeled on the top, in nM) were incubated with DNA fragments containing the promoter region of *LMANv2_200027*, *LMANv2_290065*, or *amtB* (labeled at the bottom). Probe names are indicated on the left at the position of unbound DNA, whereas bound DNA is denoted by arrows. (B) Specificity of rRpoN binding to the promoter. MRN027, MRN065, and MRN003 are the probes with mutation at the predicted σ^{54} sequence region (labeled at the bottom, far right lane, in each gel). The exclusion (−) or inclusion of 10-, 50-, and 100-fold unlabeled competitor DNA (labeled as ×10, ×50, and ×100, respectively) or the absence (−) or presence (+) of 60 nM BSA is indicated at the top of each lane. Probe names are labeled under each image. Bound DNA is denoted by arrows.

provide biochemical evidence that *amtB*, *LMANv2_200027*, and *LMANv2_290065* have an RpoN (σ^{54})-type promoter.

Since *L. interrogans* has two predicted EBPs, a gene with an RpoN (σ^{54})-type promoter can be activated either by EbpA or by EbpB. To gain biochemical evidence that *amtB*, *LMANv2_200027*, and *LMANv2_290065* are under the control of EbpA, we further performed EMSA using recombinant EbpA. Because EbpA is a putative enhancer-binding protein that can bind at a site far from the promoter, 500-bp DNA fragments of the *amtB*, *LMANv2_200027*, or *LMANv2_290065* promoter regions (EA003, EA027, and EA065, −450 and +50 bp relative to the ATG start codon) were used for the EMSAs. As shown in Fig. 6A, rEbpA was capable of binding to the upstream regions of *amtB*, *LMANv2_200027*, and *LMANv2_290065*. Unlabeled EA003, EA027, or EA065 could compete for binding, but sheared salmon sperm DNA did not. In addition, no DNA-BSA complex was observed (Fig. 6B). Furthermore, neither rRpoN nor rEbpA bound to the σ^{70}-type promoter region (L41) of *lipL41*. On the other hand, rRpoN bound to the promoter region of *lipL41* when the σ^{70}-type promoter was replaced with a σ^{54}-type promoter (RL41) (Fig. 6C). However, rEbpA could not bind to RL41 despite the fact that RL41 contained a σ^{54}-type promoter, suggesting that rEbpA binding to *amtB*, *LMANv2_200027*, and *LMANv2_290065* is specific. The results of these biochemical experiments, along with the genetic data presented above, suggest that EbpA is the regulator that activates *amtB*, *LMANv2_200027*, and *LMANv2_290065* by binding to the upstream binding regions near their promoters.

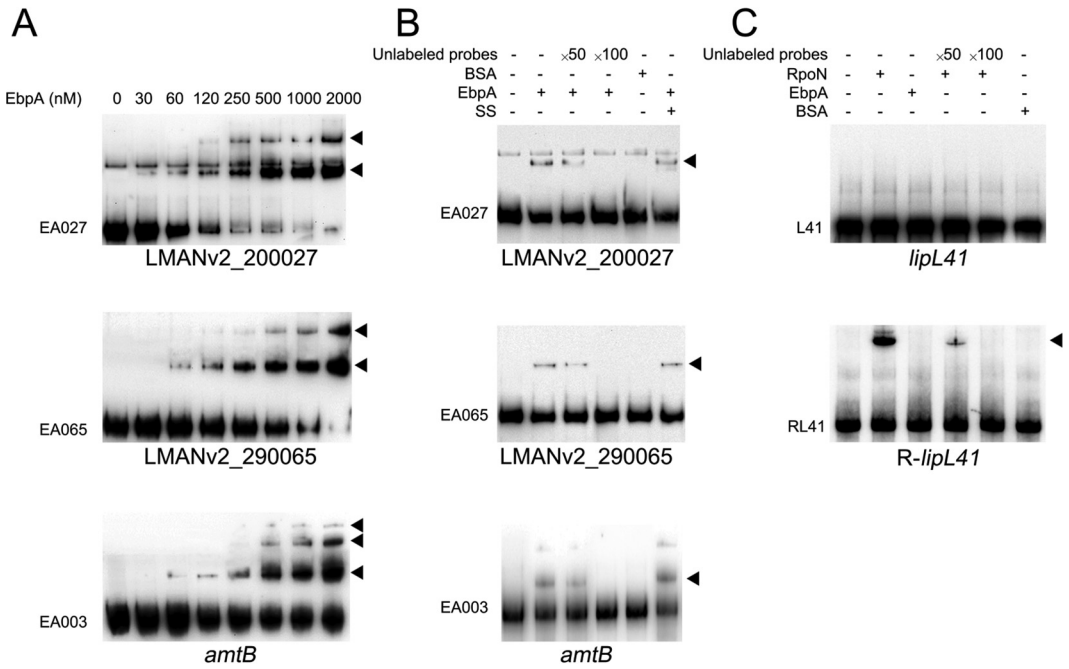

FIG 6 EMSA for the identification of EbpA-regulated genes. (A) EbpA bindings to the upstream regions of *LMANv2_200027*, *LMANv2_290065*, and *amtB*. Various concentrations of rEbpA (labeled on the top, in nM) were incubated with DNA fragments from upstream region of *LMANv2_200027*, *LMANv2_290065*, or *amtB*. Probe names are indicated on the left at the position of unbound DNA, while bound DNA is denoted by arrows. (B) Specificity of rEbpA binding to the upstream region of *LMANv2_200027*, *LMANv2_290065*, and *amtB*. The exclusion (−) or inclusion of 50- or 100-fold unlabeled competitor DNA (labeled as ×50 and ×100, respectively), the absence (−) or presence (+) of 60 nM BSA, or the absence (−) or presence (+) of 100-fold sheared salmon sperm (SS) DNA is indicated at the top of each lane. (C) Ability of rRpoN and rEbpA to bind to the promoter region of *lipL41*. Probe L41 (top panel) contains a σ^{70}-type *lip41* promoter. Probe R-*lipL41* (bottom panel) contains the *lipL41* promoter region in which the σ^{70}-type promoter sequence is replaced with a σ^{54}-type promoter.

The *ebpA* mutant has reduced growth and survival under various conditions. *amtB*, predicted to encode an ammonia transporter protein (PSORT-B, TMhmm), is putatively cotranscribed with *glnK*, which encodes a nitrogen regulatory protein in *L. interrogans* (5, 24). We therefore examined the growth of the *ebpA* mutant in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid media with alternative nitrogen sources. There was no significant difference in growth observed between wild-type and Δ*ebpA1* strains grown in standard EMJH medium with 5 mM ammonium chloride (Fig. 7A) (25, 26). However, when 5 mM L-alanine, a poor nitrogen source, was supplemented in nitrogen-free EMJH medium, the Δ*ebpA1* mutant had significantly reduced growth compared to the wild type (Fig. 7B).

To investigate the survival abilities of the Δ*ebpA1* mutant in stress conditions, wild-type, Δ*ebpA1*, and Δ*ebpA*/*ebpA*$^+$ strains were cultivated in either 1:10 diluted EMJH medium or in mineral water. The *ebpA* mutant had a lag phase at the initial stage in nutrient-limited media (Fig. 7C). Wild-type *L. interrogans* can survive in mineral water for weeks, and the spirochetal numbers remained high until week 5; the numbers began to drop at week 6 (Fig. 7D). At week 6, wild-type spirochetes remained alive and cultivable (data not shown). In contrast, the numbers of the *ebpA* mutant began to drop significantly within the first week (Fig. 7D). At week 4, although some mutant spirochetes remained (Fig. 7D), no live or cultivable mutant spirochetes could be detected (data not shown). These results suggest that EbpA is important for *Leptospira* to survive in water.

***ebpA* is dispensable for virulence.** To determine whether *ebpA* plays a role in virulence, 10^4 cells of either wild-type or Δ*ebpA2* strains were inoculated into gerbils, and the gerbils were observed for 21 days. The data indicate that the abrogation of the *ebpA* gene does not reduce mortality in gerbils (Fig. 8). Similar results were found between wild-type and Δ*ebpA1* strains when 10^6 cells were inoculated intraperitoneally

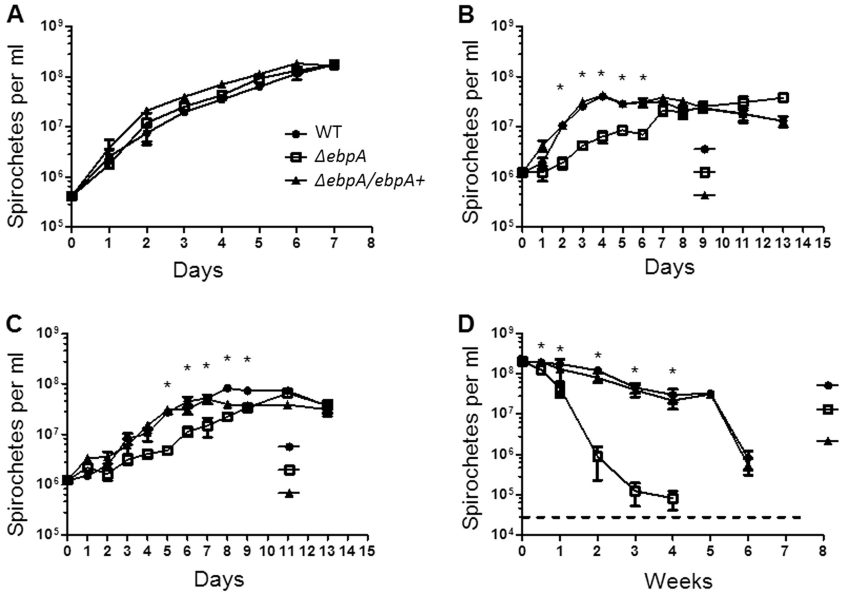

FIG 7 Growth of the *ebpA* mutant in various conditions. The wild-type (wt) *L. interrogans* strain, the *ebpA* mutant, and its complementary strain were grown to late log phase (10^7/ml) and then subcultured into regular EMJH medium (A), EMJH medium with 5 mM alanine replacing NH_4Cl (B), or 1:10-diluted regulated EMJH medium (C). (D) For the water survival test, *L. interrogans* wild type, the *ebpA* mutant, and its complementary strains were harvested by centrifugation and resuspended in mineral water. The dashed line in panel D is the limit of detection. All cultures were incubated at 30°C, and the motile leptospires were counted directly under dark-field microscopy. Error bars indicate standard errors from triplicate cultures. *, statistical differences between the wild type and the *ebpA* mutants with $P < 0.001$ (Student *t* test).

(data not shown). Viable spirochetes for all of the strains inoculated were recovered from renal tissue and blood. These results suggest that EbpA is not essential for mammalian infection.

DISCUSSION

Little is known about the signaling and regulatory pathways underlying the differential gene expression of *L. interrogans*. In this study, we provide bioinformatic, genetic, and biochemical evidence to demonstrate the presence of the EbpA-RpoN pathway in which the bacterial enhancer-binding protein EbpA and the sigma factor σ^{54} work together to activate at least three genes from their σ^{54}-type promoters: *amtB* (encoding ammonia transporter), *LMANv2_200027* (encoding a conserved exported protein), and *LMANv2_290065* (encoding a putative outer membrane protein). This pathway appears

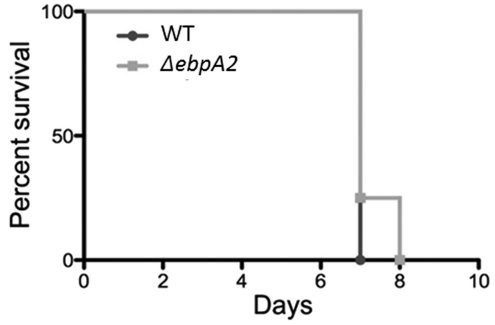

FIG 8 EbpA is dispensable for mammalian infection. Four 3-week-old male Mongolian gerbils per group were intraperitoneally inoculated with 10^4 cells of either the wild-type (WT) or the Δ*ebpA2* strain. Each group contained four gerbils, and the infectivity of each strain was reported as the percentage of animal survival.

not to be essential for mammalian infection but plays an important role for *L. interrogans* to survive in the environment.

RpoN has been shown to be involved in regulation of many bacterial functions such as nitrogen metabolism, flagellar biosynthesis, osmotolerance, pH changes, biofilm formation, motility, colonization, lipoprotein biosynthesis, and a type III secretion system (18, 27–29). Bioinformatics analysis predicted 13 putative RpoN (σ^{54})-type promoters controlling 22 genes in *L. interrogans*. Using the *ebpA* mutant and EMSA, we demonstrated that at least three of these genes—*amtB*, *LMANv2_200027*, and *LMANv2_290065*—are under the control of EbpA. Other predicted RpoN-dependent genes could be controlled by EbpB. Interestingly, three of these genes have been demonstrated to be under the control of RpoN in other bacteria: *lpxC*, which is responsible for the first committed step in lipid A biosynthesis regulated by RpoN in *Aquifex aeolicus* (30); *pstA*, encoding phosphoenolpyruvate-protein phosphotransferase, which participates in regulation of the σ^{54}-dependent promoter in *Rhizobium etli* (31); and *glnA*, encoding glutamine synthetase, the RpoN-dependent gene in *Escherichia coli* (32). Other putative RpoN-dependent genes include ones encoding a sodium bile acid symporter, a pyridoxamine-phosphate oxidase, an endoflagellar biogenesis protein, an alanine racemase, and proteins with unknown function. Although they remain to be confirmed, these observations suggest multifunctional roles of RpoN in *L. interrogans*.

We demonstrated that the *ebpA* mutant had impaired ability to use alanine as an alternative nitrogen source and, more importantly, had reduced survival in water. Bacteria assimilate a variety of inorganic nitrogen sources, such as ammonia, amino acids, peptides, polyamines and related compounds, cytosine, nucleosides, and other compounds (33). Ammonia supports the highest growth rate and is therefore considered the preferred nitrogen source for most bacteria. Ammonia and ammonium transport proteins belonging to the Amt protein family are ubiquitous in prokaryotes. Nearly all prokaryotes encode at least one Amt protein and, in some cases, two or three homologues within their respective genomes (34). Based on analyses of the genomes of leptospires, three genes were predicted to be involved in nitrogen assimilation. *amtB* (LIC10441) and *glnK* (LIC10440), cotranscribed as an operon and encoding ammonia permease and nitrogen regulatory protein II (PII), respectively (5), were dramatically downregulated in both of the *ebpA* mutant strains in our study. PII serves as a signal transduction protein for sensing external ammonium availability and nitrogen status of the cell. Ammonia permease acts as a channel for ammonium transport. In *E. coli* and other bacteria, *amtB-glnK* are also considered a signal transduction pathway. In a nitrogen/ammonia-rich environment, GlnK is physically bound to AmtB, disrupting the uptake of free ammonia (35). However, in an ammonium/nitrogen-scarce environment, GlnK is unbound from AmtB, allowing GlnK to interact intracellularly with other proteins, most of which are unknown (36, 37). In turn, this suggests that AmtB moonlights as a sensor of external nitrogen availability. Further, it has been previously suggested the AmtB-GlnK complex is critical for sensing and responding to nitrogen scarcity (33). Our data suggest a similar pattern in *Leptospira*: the reduced expression of *amtB* and *glnK* in *ebpA* mutants may create an inadequate intracellular stress response to nitrogen scarcity. There is another gene, *amt3* (LIC10589, *LMANv2_130007*), which encodes an alternative ammonium transporter that does not appear to have a RpoN-type promoter. The presence of the alternative ammonium transporter *LMANv2_130007* may explain the lack of a growth defect in the *ebpA* mutant when growing in a nitrogen-rich medium. Activation of *LMANv2_130007* or another, as-yet-unidentified ammonium transporter may work redundantly to ensure sufficient uptake of nitrogen under conditions when EbpA-RpoN is inactivated. Concordantly, previous studies have shown that expression of the *glnK-amtB* operon is generally induced during growth under limited ammonium conditions. Thus, the loss of ammonia transport, along with loss of GlnK intracellular signaling, may explain the observed growth phenotype of the *ebpA* mutant when grown with a poor nitrogen source such as alanine and mineral water (34). During infection, leptospires are exposed to a nutrient-rich host environ-

ment. It has been reported that *amtB* and *glnK* were downregulated when exposed to serum (5). This may explain why *ebpA* and *amtB* are dispensable for virulence during infection.

We found that the *ebpA* mutant has shortened cell length. This may be due to a variety of global response changes within the bacterium upon disruption of the *ebpA-rpoN* pathway. One possibility is that the lack of a strong nitrogen source in the bacterium may partially activate the stringent response pathway (*rel*) in leptospires, since *rel* activation in *Borrelia burgdorferi* is important as an alarmone response that results in global changes within that species (38). Likewise, *rel* has been associated with cell morphology changes in other bacteria (38). This phenotype was also observed in a previously described *lpxD* mutant (39). In addition to the shortened length of the *ebpA* mutant, it remains unclear whether the mutant also has an abnormal cell width, helical pitch, or flagellar length. Further study using cryo-electron microscopy will likely yield interesting findings on the nature of the morphological phenotype of the *ebpA* mutant.

We observed that the complemented strain produces higher levels of *ebpA* transcript levels than the wild type (Fig. 2), as well as for those EbpA-controlled genes (Fig. 4). One possibility is the gene dosage. This is possible but unlikely since our random nested PCR only revealed insertion into one gene (*LMANv2_590053*). Another possibility is that insertion of *ebpA* into *LMANv2_590053* has allowed the increased expression of *ebpA*. Increased expression of EbpA in turn increased the expression of EbpA-dependent genes.

In summary, we demonstrate that the presence of the EbpA-RpoN regulatory pathway is important for the environmental survival of *L. interrogans*. This pathway does not appear to be essential for mammalian survival based on our findings. The nonessential nature of EbpA-RpoN for virulence is not surprising, since the saprophytic strain *L. biflexa* also contains EbpA and RpoN. Both species may face various hostile and nutrient-scarce conditions when positioned in the natural environment (40, 41). Both species also encode other factors (e.g., σ^E) to respond to environmental stresses (42, 43). On the other hand, pathogenic *L. interrogans* encodes EbpB, which is lacking in saprophytic *L. biflexa*, where EbpB is an NtrC-type of EBP. Interestingly, *B. burgdorferi*, another spirochetal pathogen, has an NtrC-type EBP, Rrp2. Rrp2, working together with RpoN, controls the expression of RpoS, which in turn governs multiple factors required for mammalian infection (44–46). Previous research on the *ntrC* locus of pathogenic *Leptospira* has demonstrated that this locus has undergone several evolutionary rearrangements compared to other pathogenic spirochetes (47). Therefore, it is tempting to postulate that the two RpoN pathways of *L. interrogans* have different functions: EbpA-RpoN is important for environmental survival, while EbpB-RpoN may play a role in mammalian-phase adaptation.

MATERIALS AND METHODS

Strains and culture conditions. The pathogen *L. interrogans* serovar Manilae strain L495, as well as the biologically distinct Δ*LMANv2_37011* transposon mutants, i.e., the Δ*ebpA1* and Δ*ebpA2* strains (22), and the Δ*ebpA*/*ebpA*$^+$ complemented strain, were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (25, 26) at 30°C. *Escherichia coli* strains XL-10, BL21(DE3), and SM10 were grown in Luria-Bertani (LB) broth or agar with antibiotics as described below.

Construction of *ebpA* mutants and the complemented strain. *ebpA* was previously disrupted in wild-type *L. interrogans* serovar Manilae strain L495 by using the Himar1 random transposon system (22). Transposon insertion of *ebpA* in the Δ*ebpA1* mutant occurred at nucleotide 3149215 in the proximal region of *ebpA*. Transposon insertion of *ebpA* in the Δ*ebpA2* mutant occurred at nucleotide 3148873 in the proximal region of *ebpA*. The native *ebpA* allele, along with its native promoter region (including 300 bp upstream of +1), from *L. interrogans* serovar Manilae strain L495 was PCR amplified with XhoI 5′ and 3′ overhangs using the primers ebpA-x1 and ebpA-x2 (Table 2) and T-A ligated into pCRII-TOPO plasmid (Life Technologies, Waltham, MA). The resulting plasmid was transformed into XL-10-competent cells according to the manufacturer's instructions (Stratagene, La Jolla, CA). This plasmid was renamed pCRII-TOPO-*ebpA*. XL-10 cells containing pCRII-TOPO-*ebpA* were grown in 5 ml of LB broth with 50 μg of kanamycin (Sigma)/ml and 50 μg of ampicillin (Euromedex)/ml to late log phase (10^7/ml), followed by plasmid extraction by using a QIAprep spin miniprep kit (Qiagen, Valencia, CA). The *ebpA* gene was released from pCRII-TOPO-*ebpA* by 1 unit of XhoI restriction endonuclease (Thermo Scientific, Waltham, MA) and gel extracted by using a QIAquick gel extraction kit (Qiagen) according to the respective manufacturers' instructions. The gene was ligated with T4 ligase according to the manufacturer's instructions (New England BioLabs [NEB]) into the dephosphorylated XhoI site of conjugative plasmid

TABLE 2 Primers used in this study

Gene	Primer	Sequence (5′–3′)[a]	Size (bp)	Purpose
rpoN (*LMANv2_590036*)	pLATE52-rpoN F	GGTTGGGAATTGCAAATGAATCTAAGTCAGTCAC	1,419	*rpoN* gene expression
	pLATE52-rpoN R	GGAGATGGGAAGTCATTATCTAGACTCCAAAGAC		
16S (*lArRNA3064233R*)	plasmid-16S F	GCGGCGAACGGGTGAGTAACACGTGGG	968	Generation of plasmid for standard curve
	plasmid-16S R	CATCTCACGACACGAGCTGACGACAACC		
	qRT-16sRNA F	GGCGGACATGTAAGTCAGGT	179	qRT-PCR primer set
	qRT-16sRNA R	GCCTAGCGTCAGTTTTAGG		
ebpA (*LMANv2_370110*)	plasmid-ebpA F	ATGTCAGGATATGTGAAGCC	1,214	Generation of plasmid for standard curve
	plasmid-ebpA R	GCAGAGGCAATCATTTCTTTACCGG		
	qRT-ebpA F	TCCGATGATTGTTGAAGACG	262	qRT-PCR primer set
	qRT-ebpA R	GGATTTTTCCAAACGATCCA		
	ebpA-x1	CCGCTCGAGCATAGGTTTAGTGATC	2,673	Generation of plasmid for complementation
	ebpA-x2	CCGCTCGAGCGTTCACACAATCTTCTC		
	pGEX-4T-2-ebpA F	CGCGGATCCATGTCAGGATATGTGAAG	2,097	*ebpA* gene expression
	pGEX-4T-2-ebpA R	ATAAGAATGCGGCCGCATAATCGATTTT		
LMANv2_200027	plasmid-200027 F	CAAGTTTGCGGCAATTGCTCTTTTTTTAAGC	495	Generation of plasmid for standard curve
	plasmid-200027 R	CGGGTCAAATCAGTTAGAACTAGAGATGGG		
	qRT-LMANv2_200027 F	AACCAATCCCTTCATGTTCG	195	qRT-PCR primer set
	qRT-LMANv2_200027 R	AGCTTCTTCTTCCGCATTGA		
	RN027 F	GAATCTTTATAAATTT**TGGCACGCTGCTTGCA**TATATCTATACAAGAAC	49	EMSA probes for rRpoN, 3′ biotin labeled
	RN027 R	TTCTTGTATAGATATA**TGCAAGCAGCGTGCCA**AAATTTATAAAGATTCT		
	MRN027 F	GAATCTTTATAAATTT**aaaatCGCTGCaattA**TATATCTATACAAGAAC	49	EMSA probes for rRpoN, 3′ biotin labeled
	MRN027 R	TTCTTGTATAGATATA**TaattGCAGCGatttt**AAATTTATAAAGATTCT		
	FA027 F	TATCTTGCTTCGAAAGTTACC	500	EMSA probes for rEbpA, 5′ biotin labeled
	FA027 R	AGTCCGGTAAAAATGCTTAAA		
LMANv2_290065	LMANv2_290065 F	CGTCACAACTGCTCCTTTCA	216	Generation of plasmid for standard curve and qRT-PCR primer set
	LMANv2_290065 R	GCCATTGCTGCTTAGAAGG		
	RN065 F	TCTAAATTTTAAACTT**TGGCATATATTTTGCA**TAGTCTGAGCATGGAGA	49	EMSA probes for rRpoN, 3′ biotin labeled
	RN065 R	CTCCATGCTCAGACTA**TGCAAAATATATGCCA**AAGTTTAAAATTTAGAA		
	MRN065 F	TCTAAATTTTAAACTT**aaaatTATATTaattA**TAGTCTGAGCATGGAGA	49	EMSA probes for rRpoN, 3′ biotin labeled
	MRN065 R	CTCCATGCTCAGACTA**TaattAATATAatttt**AAGTTTAAAATTTAGAA		
	FA065 F	GGAACTCATACTTTTAGAAAA	500	EMSA probes for rEbpA, 5′ biotin labeled
	FA065 R	ACCAGCAGACAAAAAACAGAA		
amtB (*LMANv2_310003*)	plasmid-310003 F	GGGACTTCTTGTAAGTCCGGTATGCTACGC	538	Generation of plasmid for standard curve
	plasmid-310003 R	CTTGGTATTCGTGGCCTCTGAAAACTTCCG		
	qRT-LMANv2_310003 F	GTTAGGCGCGGTTCTTACAG	219	qRT-PCR primer set
	qRT-LMANv2_310003 R	TCTCCGTGAATTTCCGAATC		
	RN003 F	ATTATTCTTCAAT**TGGTACTAATCTTGCA**AACAAAGAATAAAAATTGAT	49	EMSA probes for rRpoN, 3′ biotin labeled
	RN003 R	TCAATTTTTATTCTTTGTT**TGCAAGATTAGTACCA**ATTGAAGAATAATT		
	MRN003 F	ATTATTCTTCAAT**aaaatCTAATCaattA**AACAAAGAATAAAAATTGAT	49	EMSA probes for rRpoN, 3′ biotin labeled
	MRN003 R	TCAATTTTTATTCTTTGTT**TaattGATTAGatttt**ATTGAAGAATAATT		
	FA003 F	TATATTTTATAAGCATACTTT	500	EMSA probes for rEbpA, 5′ biotin labeled
	FA003 R	GTGCTAGCTTCCTGCCCAAAT		

(Continued on following page)

TABLE 2 (Continued)

Gene	Primer	Sequence (5′–3′)[a]	Size (bp)	Purpose
lipL41	L41/RL41 F	TTGTAATTCAGTATCTTG	221	EMSA probes for rRpoN or rEbpA, 5′ biotin labeled
	L41/RL41 R	AAGAACATAAGGAGAACT		
raiA (*LMANv2_200026*)	q-LMANv2_200026 F	CCGCTGAAAAATACGCAAAT	193	qRT-PCR primer set
	q-LMANv2_200026 R	TCTTCCAGACCGTCGATACA		
lpxC (*LMANv2_430009*)	q-LMANv2_430009 F	CGGCAGAACTCAGCAATGTA	162	qRT-PCR primer set
	q-LMANv2_200026 R	CGTCCATGATCGGAACTTCT		
secE (*LMANv2_260002 F*)	q-LMANv2_260002 F	GCAGAATTGGAAAAGGTCCA	189	qRT-PCR primer set
	q-LMANv2_260002 R	CTAACCAGCCACTTCCCAAA		
glnA (*LMANv2_500019*)	q-LMANv2_500019 F	TTGCGGATACCGCTTATTTC	170	qRT-PCR primer set
	q-LMANv2_500019 R	AGTACCAGGACGGTGACCAG		
glnK (*LMANv2_310004*)	q-LMANv2_310004 F	AAGCGACGTTCAAGGTTACG	249	qRT-PCR primer set
	q-LMANv2_310004 R	GCGGAACTTCCTCTTTCTCC		
nctP1 (*LMANv2_210009*)	q-LMANv2_210009 F	TCCCGCAAATTTGAAAAGAC	208	qRT-PCR primer set
	q-LMANv2_210009 R	TCGACGTTCTTTGTGGATGA		
pdxH (*LMANv2_210047*)	q-LMANv2_210047 F	GTTAGCCTCACCGCAAAGTC	178	qRT-PCR primer set
	q-LMANv2_210047 R	ATCGTGTAAACGGCTGGAAC		
LMANv2_240139	q-LMANv2_240139 F	TTCCGGTCCAGAGACTTTTG	250	qRT-PCR primer set
	q-LMANv2_240139 R	GATCGAAACGTTTTCGGAGA		
LMANv2_240051	q-LMANv2_240051 F	TTTGTTTGCCCGGATAAAAG	151	qRT-PCR primer set
	q-LMANv2_240051 R	TTTTCCGAAATCGATCCAAG		
LMANv2_300019	q-LMANv2_300019 F	TTCGTGTTTGATCGTTTCCA	248	qRT-PCR primer set
	q-LMANv2_300019 R	CCTTTCCGGCAAAAAGTAGA		
fliO (*LMANv2_150090*)	q-LMANv2_150090 F	GGAATCGCAGGGACTCTTCT	217	qRT-PCR primer set
	q-LMANv2_150090 R	CATTGTCAGCAACTCCGAGA		
LMANv2_90055	q-LMANv2_90055 F	TGTATTGAAGCGGGGATTTC	184	qRT-PCR primer set
	q-LMANv2_90055 R	TACGAGCTGTTTCCGGTCTT		

[a]Restriction endonuclease cut sites are underlined. The sequences in boldface are the predicted binding sites for RpoN. Lowercase nucleotides are the mutated sites.

pAL614 carrying a modified Himar1 transposon element, along with an *aadA* (spectinomycin resistance) cassette (a gift from Ben Adler). The resulting plasmid was renamed pAL614-*ebpA*. It was transformed into *E. coli* BL21(DE3) and grown in LB broth with 0.3 mM diaminopimelic acid (Sigma) and 50 μg of spectinomycin (Sigma)/ml as previously described (48). The *ebpA* construct was then randomly *cis* inserted into the genome of the Δ*ebpA1* mutant (Man250) via conjugation between Man250 and *E. coli* strain SM10 carrying pAL614-*ebpA* as previously described (39). Sequence analysis was performed as previously described (39) and revealed that the Δ*ebpA*/*ebpA*$^+$ clone used in this study was *cis* inserted into *LMANv2_590053*.

RNA isolation and qRT-PCR. For the initial qRT-PCR analysis of wild-type and Δ*ebpA1* samples by the relative quantity, leptospires were grown in EMJH medium at 28°C and harvested when bacterial growth reached the late logarithmic phase (10^7/ml). Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen), and genomic DNA was digested using RNase-free DNase I (Promega). RNA was then further purified by using an RNeasy minikit (Qiagen). cDNA was synthesized using the SuperScript III reverse transcriptase with random primers (Invitrogen, Carlsbad, CA). Using the cDNAs as the templates, the mRNA was assessed by real-time fluorescence quantitative PCR (qPCR) using a SYBR green PCR master mixture (ABI, Pleasanton, CA) in an ABI 7000 real-time PCR system (ABI). The primers used in the qPCR are listed in Table 2. In the qRT-PCR, the 16S rRNA gene of *L. interrogans* serovar Manilae strain L495 was used as the internal reference. The qRT-PCR data were analyzed using the $\Delta\Delta C_T$ model and the randomization test in the REST 2005 software. For subsequent qRT-PCR analysis of wild-type, Δ*ebpA1*, Δ*ebpA2*, and Δ*ebpA*/*ebpA*$^+$ strains, RNA was generated from strains grown in EMJH medium with 1% rabbit serum until reaching late logarithmic phase as described above. cDNA was generated by using 1 μg of triple-DNase-treated RNA with the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad). Next, 2 μl of 1:10-diluted cDNA was added to SsoFast Evagreen Supermix, along with 250 nM concentrations of the primers, according to the manufacturer's instructions (Bio-Rad). qPCR was performed using a CFX 96 real-time system/C1000 thermal cycler (Bio-Rad) with an amplification program of 1 cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and a plate read, then 1 cycle at 95°C for 10 s, and finally followed by melting-curve analysis of 65 to 95°C in 0.5°C increments for 5 s each. Bio-Rad CFX Manager v3.1 was used to determine the quantification cycle threshold (C_T), and the absolute quantity (ΔC_T) was determined by comparing copy number of each gene to copy number of 16S. Statistical analysis was completed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test, where significance was defined as $P < 0.05$.

Standard curves were generated for the 16S rRNA gene (*LArRNA2417572R*), *ebpA* (*LMANv2_370110*), *LMANv2_200027*, *LMANv2_290065*, and *LMANv2_310003* (*amtB*) using the primers described in Table 2. Each cassette was amplified from *L. interrogans* serovar Manilae strain L495 by PCR as described above, T-A ligated into pCRII-TOPO plasmid (Life Technologies), and transformed into XL-10 Gold- or XL-1 Blue-competent cells (Stratagene) according to the manufacturer's instructions. Cells were subsequently grown in 2 ml of LB broth with 50 μg of kanamycin/ml and 50 μg of ampicillin/ml until reaching the late logarithmic phase (10^7/ml) and plasmid extracted by QIAprep spin miniprep kit according to the manufacturer's instructions (Qiagen). The plasmid concentration was calculated by spectrophotometric analysis ($A_{260/280}$) by using a NanoDrop Lite apparatus (Thermo Scientific). The total copy numbers of each plasmid were then calculated using the URI Genomics and Sequencing Center tool (http://cels.uri.edu/gsc/cndna.html), and a standard curve was generated.

Recombinant protein expression and purification. Recombinant RpoN was produced in *E. coli* using the aLICator LIC cloning and expression system (Thermo Scientific). Briefly, the full-length *rpoN* gene was amplified from *L. interrogans* serovar Manilae strain L495 using the primers pLATE52-rpoN F and pLATE52-rpoN R (Table 2) and cloned into pLATE52 to generate the pLATE52^{rpoN} plasmid, which was then transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen) for sequencing. The plasmid containing the *rpoN* sequence was transformed into *E. coli* BL21(DE3) (Novagen) to form *E. coli* BL21(DE3)/pLATE52-rpoN. All of the engineered bacteria were cultured in LB broth containing 100 μg of ampicillin/ml to express the recombinant proteins under induction by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma). The His_6-tagged recombinant proteins were purified using the Ni-NTA column according to the manufacturer's instructions (Qiagen) and analyzed by SDS-PAGE. To obtain the recombinant protein EbpA, the full-length *ebpA* gene was PCR amplified from *L. interrogans* strain L495 genomic DNA using the primers pGEX-4T-2-ebpA F and pGEX-4T-2-ebpA R (Table 2). The product was cloned into pMD18-T to form a recombinant pMD18-T^{ebpA} plasmid for sequencing. The pMD18-T^{ebpA} and pGEX-4T-2 vectors (Amersham Pharmacia Biotech) were digested with both BamHI and NotI endonucleases (NEB). The recovered *ebpA* gene segment was linked to the linearized pGEX-4T-2 using T4 DNA ligase (NEB) to form the pGEX-4T-2^{ebpA} plasmid. pGEX-4T-2^{ebpA} was transformed into *E. coli* BL21(DE3) (Novagen) to generate *E. coli* BL21(DE3)/pGEX-4T-2^{ebpA}. The recombinant EbpA protein (rEbpA) was expressed by induction with 0.5 mM IPTG (Sigma), and the fusion protein with glutathione *S*-transferase (GST) was extracted from the cell lysates after ultrasonication using Bio-Scale Mini Profinity GST Cartridges (Bio-Rad). The rEbpA protein was cleaved with thrombin (Amersham Pharmacia Biotech) and purified further with an Amicon Ultra-4 centrifugal filter (Millipore; nominal molecular size limit, 50 kDa) to remove the GST and thrombin. The soluble recombinant protein was filtered by using a 0.22-μm-pore-size membrane filter, examined by SDS-PAGE, and measured by using a BCA protein assay kit (Bio-Rad).

EMSA. The primers used in the EMSAs are listed in Table 2. Synthesized or PCR-amplified DNA probes were end labeled with biotin using biotin-labeled forward primers (Invitrogen, Shanghai, China). Each EMSA was performed by using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). Briefly, a 200 nM concentration of labeled probe was mixed with various amounts of purified rRpoN or rEbpA in 20 μl of the gel shift binding buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 5%

(vol/vol) glycerol. After incubation at 37°C for 30 min, the samples were analyzed by 10% nondenaturing polyacrylamide gel electrophoresis at 60 V for 3 to 6 h. The DNA was then transferred onto a positively charged nylon membrane (Roche Applied Science, USA) by electroblotting (380 mA for 80 min). After UV cross-linking, blotting, and washing, the membrane was conjugated by using stabilized streptavidin-horseradish peroxidase. The signals were detected according to the manufacturer's instructions after exposure using a camera equipped with a charge-coupled device imager (FluorChem E; ProteinSimple).

Growth curve, survival, and cell morphology. *L. interrogans* strains, including the *ebpA1* mutant, were first grown in standard EMJH medium to late log phase (10^7/ml). The bacterium-containing medium was then diluted at a ratio of 1:10 into EMJH medium or into EMJH medium with 5 mM alanine replacing the NH_4Cl. For the water survival test, *L. interrogans* strains were grown to late logarithmic phase before they were harvested by centrifugation. The supernatants were drained thoroughly, and the cells were resuspended to the original volume in mineral water (pH 7.2; Kirkland Signature). All cultures were incubated at 30°C, and the motile leptospires were enumerated every 24 h under dark-field microscopy by using a Petroff-Hausser counting chamber according to the manufacturer's instructions (Hausser Scientific, PA). The viability of the spirochetes was confirmed by growth in standard EMJH medium.

Cell morphology was measured in wild type, Δ*ebpA1*, Δ*ebpA2*, and Δ*ebpA*/*ebpA*$^+$ cells grown in standard EMJH medium to late logarithmic phase (10^7/ml) as described above. The lengths of 100 to 200 cells of each strain were measured by using an Olympus Bx53 microscope and Olympus CellSens Dimension 1.7.1 software (Olympus, Hamburg, Germany). Fields were randomly selected for measuring leptospire cell length. The average lengths and the standard errors of the mean were calculated by using Prism software (GraphPad, San Diego, CA). Statistical analyses were completed by one-way ANOVA and Tukey's HSD test, where significance was defined as $P < 0.05$.

Determination of the contribution of EbpA to virulence in Mongolian gerbils. Three-week-old male Mongolian gerbils (*Meriones unguiculatus*) were purchased from Janvier labs (Janvier, Le Geneste-Saint-Isle, France). Four gerbils per group were tested for survival against wild-type, Δ*ebpA1*, or Δ*ebpA2* strains in accordance with the guidelines of the Animal Care and Use Committees of the Institut Pasteur (authorization B-75-1132). After intraperitoneal injection with 10^4 or 10^6 leptospires, the gerbils were observed twice daily until day 21 postinoculation. After euthanasia at day 21, approximately 300 μl of blood was collected, along with the kidneys by dissection. The outgrowth of spirochetes in these tissue cultures was assessed using dark-field microscopy.

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